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Dedication

For my parents, Martin and Donna Tepe

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List of Abbreviations

α-MHC	α-Myosin Heavy Chain
AC	Adenylyl Cyclase
ACV	Adenylyl Cyclase Type V
ANF	Atrial Natrietic Factor
A-R-G	Agonist-Receptor-G-protein Complex
βAR	β-Adrenergic Receptor
β_1AR	β_1 subtype of the βAR
β_2AR	β_2 subtype of the βAR
βARK	β-Adrenergic Receptor Kinase
βARK1	β-Adrenergic Receptor Kinase, Type 1
cAMP	3',5'-cyclic monophosphate
CHF	Congestive Heart Failure
ECL	Enhanced Chemiluminescence
FS	Fractional Shortening
G-Protein	GTP binding Protein
Gi	Inhibitory G Protein
Giα_2	α subunit of type 2 Gi protein
Gq	PLC Coupled G Protein
Gαq	α subunit of Gq protein
Gs	Stimulatory G Protein
GRK	G-Protein Coupled Receptor Kinase
GRK2	G-Protein Coupled Receptor Kinase, Type 2
GRK3	G-Protein Coupled Receptor Kinase, Type 3
GRK5	G-Protein Coupled Receptor Kinase, Type 5
GTP	Guanine Trisphosphate
HEK cells	Human Embryonic Kidney Cells
I_{Ca}	Calcium Current
ICYP	Iodo-cyanopindolol

K_H	Affinity; high-affinity receptor site
K_L	Affinity; low-affinity receptor site
mRNA	messenger RNA
NaF	Sodium Fluoride
PDE	Phosphodiesterase
PKA	protein kinase A
PKC	protein kinase C
PLC	Phospholipase C
R_L	proportion of receptors in high affinity state
R_H	proportion of receptors in low affinity state
RIA	radioimmunoassay

Abstract

Cardiac overexpression of the GTP binding protein Gαq five-fold over background in mice results in a phenotype that includes depressed *in vivo* responsiveness to infused β-adrenergic receptor agonists. This model was used to explore the mechanisms of β-adrenergic receptor (βAR) regulation in cardiac hypertrophy and contractile dysfunction.

Studies were carried out investigating receptor number, coupling efficiency, and downstream components of the pathway including inhibitory and stimulatory G proteins, kinases and adenylyl cyclase (AC). A number of key biochemical findings in myocardial membranes or intact myocytes pointed towards potential mechanisms imparting various aspects of the dysfunctional βAR phenotype. At the receptor level, there were: no changes in total βAR number, a decrease in high affinity βAR, and a decrease in basal and β_1AR and β_2AR stimulated adenylyl cyclase activity. Two of the three classes of kinases known to modulate βAR were found to be altered, with PKCα expression increased, βARK decreased and PKA unchanged. At the G protein level, Gi_2 and Gi_3 were both increased and Gs was unchanged. At the G protein/adenylyl cyclase interface, markedly depressed responsiveness to NaF and forskolin occurred, and a decrease in the predominant AC (Type V) was found.

To assess the contribution of increased Gi to the overall phenotype, pertussis toxin was used *in vivo,* in isolated myocytes, and a model transfected cell system was used to determine the effects of Gi on adenylyl cyclase

activation. Results of these studies suggested that increased Gi has a small contribution to the aberrant signalling in the Gαq animal.

Transgenic models were used to assess the contribution of decreased receptor signalling and the observed decrease in adenylyl cyclase protein/activity. Overexpression of moderate levels of the β_2AR or Type V adenylyl cyclase in an otherwise normal background was tolerated without detrimental effects. Overexpression of the β_2AR in the Gαq background resulted in attenuated development of hypertrophy and an improved basal cardiac function at lower levels but higher levels of receptor were detrimental to function. Overexpression of Type V adenylyl cyclase in Gαq animals at levels that completely restored forskolin stimulated activity resulted in normalized βAR signalling and improved basal contractility, but did not reverse hypertrophy, identifying a critical role of AC downregulation in the Gαq phenotype.

Chapter 1

Introduction

A hallmark of congestive heart failure observed to occur following cardiac hypertrophy is a depressed responsiveness to catecholamines (Bristow, 1982). The molecular basis of this altered responsiveness, which is thought to contribute to the symptoms and pathophysiology of the syndrome and may also provide a long term protective effect, is not well understood. The primary goal of these studies is to characterize βAR signalling in a transgenic mouse model in order to delineate potential molecular mechanisms of hyporesponsiveness in hypertrophy and to identify specific contributions of these defects to the compromised phenotype. To investigate these changes, a murine model of cardiac hypertrophy was primarily utilized. This model is a transgenic mouse that overexpresses the Gαq subunit of the Gq-type GTP binding protein. The mouse exhibits eccentric cardiac hypertrophy and compromised LV function with many features of experimental and human heart failure. (D'Angelo et al. 1997). Hearts of Gαq animals are unresponsive to isoproterenol *in vivo*, providing an ideal model for investigation of impaired adrenergic signalling.

Limited observations of the human failing heart have suggested multiple potential changes that occur in heart failure regarding the βAR pathway: the β_1 adrenergic receptor subtype has reduced expression (Bristow, 1986), Gi protein levels are increased (Feldman, et al), βARK (a G-protein

coupled receptor kinase) is upregulated (Ungerer 1994), and the coupling of both β_1 and β_2 receptors to adenylyl cyclase is impaired (Bristow, et al. 1982). However, this work is primarily based on investigation of end-stage failing human hearts, is largely observational in nature, and is typically limited to the availability of tissue. Such approaches cannot address primary versus secondary changes and do not allow for delineating mechanism. In addition, this work lacks the ability to assign the contribution of a specific defect to the phenotype.

To address these issues, the signalling properties of the Gαq model were characterized and candidate biochemical mechanisms delineated. To determine the importance of these mechanisms in the development of the pathologic, physiologic and biochemical characteristics of the Gαq mice, dual transgenic mice were created in an effort to improve different aspects of the phenotype. This allowed for assignment of specific phenotype-lesion relationships.

Finally, the role of the catalytic unit of the signalling pathway, adenylyl cyclase (AC), was investigated independently, and in the context of Gq mediated dysfunction. It has been proposed that the expression levels of this cAMP generating component limit cardiac βAR function under normal physiological conditions, and that overexpression of AC will enhance βAR signalling (Gao, 1998). To more clearly understand this critical aspect of receptor-effector signalling, animals were created which overexpress the type

V isoform of adenylyl cyclase approximately 50% over NTG levels. These animals were studied independently, and were crossed with the Gαq mouse to identify the contribution of this protein to βAR mediated contractile dysfunction.

Chapter 2

Background

Each year, 400,000 new cases of heart failure are diagnosed, the majority being either due to ischemic cardiomyopathy or idiopathic dilated cardiomyopathy. The 5 year survival rate for those with heart failure is 50% and is considerably less for those with severe symptoms (Kannel, et al 1991; Ho, et al 1993). Despite extensive research and the advancements of technology, the molecular basis of hypertrophy and heart failure remains undetermined. Although drugs have been developed to help alleviate the symptoms of heart failure, less progress has been made in the development of agents that significantly reverse the process primarily because of a lack of understanding of the mechanisms of ventricular dysfunction and its progression to failure.

Cardiac Hypertrophy and Progression to Failure

Cardiac hypertrophy is the physiological response that allows the heart to compensate for increased hemodynamic stress via an increase in myocyte size. The resulting increase in myocardial mass and wall thickness decreases wall stress, thereby normalizing stroke volume. However, this compensation is only maintained for a finite period of time, as this condition paradoxically increases metabolic demand. Ultimately, the heart begins to dilate, the walls become thinner, wall stress increases, and myocyte number decreases via apoptosis (programmed cell death). Hypertrophy has been identified as a significant risk factor for cardiovascular events such as

congestive heart failure, myocardial infarction, and sudden death. (Messerli, et al. 1995, Vasan , et al. 1997), and its treatment with ACE-inhibitors, diuretics, calcium channel blockers or beta-blockers can significantly reduce these cardiovascular complications (Schmieder, et al. 1997).

Overview of the βAR system in the heart

In the heart, the positive chronotropic and inotropic effects of endogenous and exogenous catecholamines are mediated by the β-adrenergic receptors. Cardiac tissue expresses both the β_1 and β_2 subtype. Of the β receptors in the heart, approximately 70% are of the β_1 subtype and 30% are of the β_2 subtype (Minneman, 1981, Heitz, 1993). β–adrenergic receptors are seven transmembrane spanning proteins that signal through the activation of adenylyl cyclase. (Figure 1 depicts a simplified diagram of the βAR pathway.) Both β1 and β2 receptor subtypes couple to a heterotrimeric stimulatory GTP-binding protein, Gs, composed of an α, β and γ subunit. Activation of Gs by agonist occupied receptor results in dissociation of α subunit from the βγ subunits. The α subunit is the GTP binding subunit of the G protein. The GTP-bound α subunit is responsible for the stimulation of membrane bound adenylyl cyclase. Activated adenylyl cyclase catalyzes the conversion of ATP to cAMP which then activates protein kinase A (PKA) by binding to the regulatory subunits. Activated PKA then phosphoryates key intracellular proteins such as troponin I, phospholamban and L-type calcium channels, resulting in enhanced cardiac function. Release of βγ subunits

during activation of G proteins can also result in activation of certain adenylyl cyclase isoforms, the MAP kinase pathway, a muscarinic potassium channel, and translocation of G protein coupled receptor kinases.

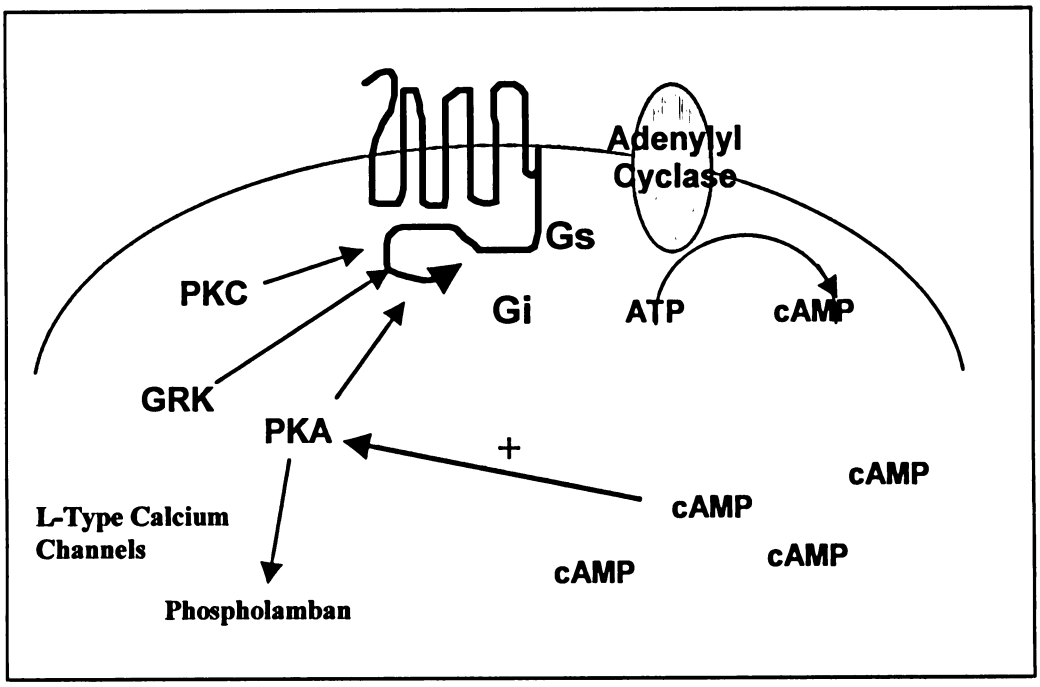


Figure 1: Simplified diagram of the βAR pathway

The adrenergic receptors are regulated through events that serve to either uncouple the receptor from Gs or reduce receptor number at the membrane. Uncoupling occurs when agonist-occupied receptor is phosphorylated by intracellular kinases, resulting in an inhibition of receptor-G protein interactions. This phosphorylation can be mediated by three distinct types of cellular kinases: the G-protein coupled Receptor Kinases (GRKs), protein kinase A (PKA), and protein kinase C (PKC) (Freedman, et al. 1995, Lohse, et al., 1996, Pitcher, et al., 1992, Bouvier, et al., 1991). GRK mediated desensitization is termed "homologous desensitization", as only

receptor occupied by agonist is phosphorylated and uncoupled from its effector. The GRK family of proteins is comprised of six isoforms. These proteins are critical regulators of the βAR system and have been implicated in the development of contractile dysfunction. GRKs are primarily cytosolic, with the exception of GRK5, which is membrane bound. When activated, the cytosolic GRK protein is translocated to the membrane by βγ subunits. This translocation allows the kinase to phosphorylate the receptor. The GRKs desensitize the receptor in combination with proteins called β-arrestins. β-arrestins associate with the GRK phosphorylated receptor to facilitate uncoupling from its effectors (Ferguson, et al. 1996). PKA or PKC mediated desensitization, alternatively, is termed "heterologous desensitization". These kinases can phosphorylate both agonist occupied and unoccupied receptors when activated, and can thus be a mechanism whereby other activated receptors can desensitize βAR. PKC has also been demonstrated to activate some GRK isoforms (Pronin, et al., 1997, and DeBlasi, et al. 1995) increasing the overall kinase activity. Downregulation of the receptor, in contrast to desensitization via phosphorylation, is a decrease in receptor number at the cell surface. This occurs as a result of decreased synthesis and/or enhanced degradation of the receptor in response to prolonged agonist exposure.

As noted above, G proteins, which couple the receptor to downstream effectors, are a class of hetertrimeric proteins that bind GTP. The three subunits that compose this protein are termed α, β and γ. Inactive G protein is bound to GDP. Upon receptor activation, G proteins release

GDP and bind GTP, and the βγ subunits dissociate from the α subunit. This α subunit then regulates the activities of effectors downstream of the receptor. (Watson and Arkinstall, 1994). The actions of the α subunit are specific to the class of G protein. There are several classes of G proteins. These include Gs, Gi, Gq and G_{12}. The Gs protein activates adenylyl cyclase, increasing the concentration of cAMP within the cell, whereas Gi protein inhibits this enzyme. Gq protein activates phospholipase C (PLC) which hydrolyzes PIP_2 to IP_3 and diacylglycerol. IP_3 then binds to receptors on the sarcolemma initiating the release of intracellular stores of calcium. Diacyglycerol is a second messenger that activates some isoforms of protein kinase C (PKC). The G_{12} class of G proteins is not as well characterized, but has been described to play a role in the regulation of mitogenic pathways, the Na^+/H^+ exchanger and transcriptional regulation of primary response genes (Dhanasekaran, et al. 1996).

Adenylyl cyclase is the catalytic moiety in the βAR pathway responsible for the generation of cAMP. Regulation of this enzyme may play a key role in the development of heart disease and refractoriness to catecholamines. This protein consists of a tandemly repeated six transmembrane spanning domain with a large cytoplasmic loop. (See Figure 2.) This structure resembles that of an ion channel or transporter. To date, nine isoforms of adenylyl cyclase have been cloned, with many having tissue specific distribution. In the heart, types V and VI are the major isoforms (Ishikawa and Homcy 1997), although message for types II, IV, and VII can

be detected. These two major isoforms, V and VI, appear to be differentially expressed in cardiac tissue. In a study of chick myocytes, the amount of type V adenylyl cyclase was found to be 4-5 times that of type VI, while the signal for type VI was stronger in non-myocytes. (Yu et al., 1995). Although this localization has not been confirmed in other species, it is likely that type V is a myocyte specific isoform. This is supported by a more recent study that examined the mRNA levels of these isoforms in human heart tissue. Using cardiac homogenates, type V was found to be in 60 fold excess of type VI, suggessting that this is the predominant human cardiac isoform (Raimundo, et al 1999). All of the adenylyl cyclase isoforms are activated by Gs. Only types II, V and VI are inhibited by Gi, though the degree of inhibition may vary among the isoforms. (Iyengar, 1993). Types V and VI are inhibited by uM concentrations of Ca^{2+} (Yoshimura and Cooper, 1992). (Types I and III appear to be stimulated by calcium, whereas types II and IV are not affected). Despite the ubiquitous nature of this effector protein, much still remains to be discovered concerning the isoform specific regulation of the various adenylyl cyclases.

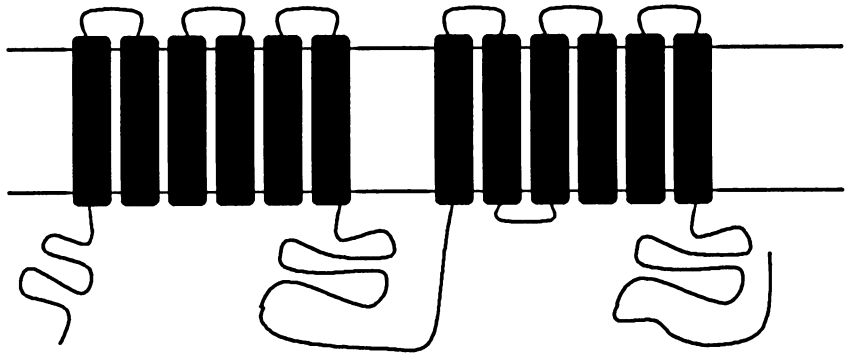


Figure 2. Proposed topology of Adenylyl Cyclase Type V

Human Studies involving the role of the βAR in Contractile Dysfunction

The initial observation by Bristow et al. (1982) that β–agonist mediated contractility is blunted in the failing heart led to investigation of the molecular components of the βAR system. Impaired contractility through this pathway was postulated to result from changes either in components of the signalling pathway, or in the contractile apparatus of cardiac myocytes.

There are multiple components in the pathway that could contribute to impaired βAR agonist responsiveness. Bristow et al. (1982) first investigated the molecular components of the βAR system in heart failure. His work, using surgically obtained failing and nonfailing hearts, showed that agonist stimulated adenylyl cyclase activity and total β-adrenergic receptor density was significantly reduced in the failing hearts. Later work revealed that this decrease in receptor density was due to selective downregulation of the β_1AR, and that the β_2AR density was unchanged. The significance of this finding was confirmed with the discovery that the degree of downregulation

of the receptor corresponded with the severity of heart failure (Fowler et al., 1986). In addition to selective downregulation, both receptor subtypes were found to have impaired functional coupling to adenylyl cyclase. (Bristow et al., 1989).

Other components of the pathway, including G-proteins, kinases and adenylyl cyclase have been demonstrated to be altered in hypertrophy and human heart failure. In work done by Ungerer et al. (1993), it was found that β–agonist stimulation of failing human heart tissues was blunted compared to control tissues, whereas calcium and forskolin stimulation were unchanged. This suggested that the defect was upstream of adenylyl cyclase and did not involve a defect in the contractile apparatus of the cell. This was thought to be caused by a combination of receptor downregulation and uncoupling from its effectors. As phosphorylation by βARK and subsequent binding by β-arrestin is a major mechanism by which β-receptors are uncoupled, GRK activity and βARK mRNA levels were assessed. βARK mRNA levels were increased almost threefold and βARK activity was increased in failing human heart samples compared to non-failing control hearts. Thus, it was established that the defect was indeed multi-factorial, involving multiple components of the signalling cascade.

Subsequent to the finding that βARK protein was upregulated in heart failure, it was postulated that an upregulation of the complementary protein, β-arrestin, may also occur. This was investigated by Ungerer et al.

(1994), who determined that neither mRNA nor protein levels of β-arrestin 1 and β-arrestin 2 were altered in heart failure, despite a threefold upregulation of βARK 1, and a slight (less than a 50%) increase in βARK 2.

Feldman et al. (1988) continued investigation of the βAR pathway in heart failure with studies of the opposing G proteins in the heart, Gs and Gi. Studies of human failing hearts revealed a 36% increase in the levels of Gi (as assessed using pertussis toxin-catalyzed ADP ribosylation) and no change in the levels of Gs. The significance of this finding, however, is difficult to speculate, as in a given cell type the levels of Gi are generally in excess of Gs and are not limiting in number. However, it cannot be assumed that the cellular processes that regulate contractility and responsiveness to catecholamines are not sensitive to subtle changes in the ratio of Gs to Gi, and the contribution of such a change is deserving of further investigation.

Despite general agreement that a significant reduction in βAR density occurs in the failing heart, there have been conflicting results concerning the integrity of the adenylyl cyclase moiety. In failing human hearts, it is notable that the positive inotropic effects of phosphodiesterase (PDE) inhibitors are also blunted. (Schmitz et al., 1989), suggesting a defect in basal cAMP production independent of receptor activation. This occurs without a change in affinity of the target enzymes (PDEs) for inhibitor. Decreased basal cAMP levels could result from defects at several key interfaces in the βAR pathway. One possibility is a decrease in the number of

receptors in the so-called R*, (or active), conformation which contributes to basal activity of adenylyl cyclase, or an alteration in the relative ratio of Gi to Gs, thereby affecting tonic regulation of adenylyl cyclase by G proteins. Alternatively, a defect in the effector protein, adenylyl cyclase, could result in reduced basal cAMP levels. Two methods of stimulating adenylyl cyclase by non-receptor means involve the use of sodium fluoride (which activates Gs and Gi) and forskolin (which directly activates adenylyl cyclase). Use of these agents in studying failing hearts has yielded different results. Work by Bristow et al. (1982) did not show a difference between NaF stimulated cyclase activity in failing versus non-failing hearts. However, in patients with congenital heart disease, receptor-independent adenylyl cyclase stimulation by forskolin was reduced by 52% in severely failing hearts compared to control. (Reithmann et al., 1997). This finding suggests that decreased adenylyl cyclase protein could be a contributing factor in the compromised human heart.

βAR Signalling in Animal models of Hypertrophy and Heart Failure

The use of animal models is critical to the investigation of contractile dysfunction. Heart tissue from human sources in not readily available, and is, in general, limited to that of end-stage failing hearts. Thus, the most readily available alternative is genetically engineered or mechanically induced models of heart failure. Such animal models have allowed for more rapid determination of changes in the βAR pathway that occur in contractile dysfunction and hypertrophy. Although animal models

may not exactly reproduce the biochemical events that occur in human conditions, these models allow identification of molecular interactions and protein alterations that contribute to or cause dysfunction.

Mechanically induced heart failure and hypertrophy can be achieved via aortic constriction (causing a pressure overload) or ventricular pacing. These methods provide cardiac tissue that allows exploration of the changes that occur in the β-adrenergic pathway with development of dysfunction. One such example of an animal model used to study cardiac dysfunction is the pacing induced canine model of congestive heart failure (CHF). In this model, pacing of the heart to 240 bpm stimulates heart failure (Ping et al., 1997) In a mildly induced form of CHF, using this model, no change in βAR number and G protein content is observed. Although there is no change in receptor number, decreased βAR coupling and reduced βAR stimulation of adenylyl cyclase (AC) is evident accompanied by significant increases in total GRK activity, protein and message. In the severe form of failure in this model, βAR downregulation and uncoupling occurs, in addition to a reduction in basal AC activity, and a reduction in expression of some AC isoforms.

In an alternate canine model (Vatner et al., 1985) pressure overload induced heart failure (achieved by aortic banding) recapitulates many of these findings. Left ventricular (LV) failure was characterized by a doubling of LV weight/body weight ratio. Basal and isoproterenol stimulated adenylyl cyclase activity was depressed in failing hearts. Receptor independent

stimulation of adenylyl cyclase activity was also severely depressed compared to control. Unlike many other heart failure models, βAR density was actually *increased*, although a two-fold *decrease* in affinity was observed. This was likely the result of a significant decrease in high affinity β receptors. In control animals, 51% of the receptors were found to exist in the high affinity state, whereas only 11% of the βAR assumed this high affinity conformation in the heart failure model.

Likewise, in the pressure overload hypertrophy in rat, (Holmer et al., 1996) left ventricular adenylyl cyclase activity in response to forskolin was significantly impaired. Forskolin, GTPγS, and manganese chloride stimulated AC activity was approximately 40% less than that of control animals, suggesting a defect in components downstream of the receptor, including G proteins and adenylyl cyclase.

Hypertrophy and failure have also been described in animal models as a result of cardiac specific transgenic manipulation. For example, cardiac specific overexpression of a constitutively active α_{1B}-Adrenergic receptor, results in hypertrophy, increased myocyte cross sectional area, and increased ANF and DAG expression. (Milano, et al, 1994).

Calsequestrin overexpression in the heart is another genetic model that results in marked left ventricular enlargement, severely depressed systolic function and decreased βAR density and βAR activated adenylyl cyclase

activity (Cho, et al. 1999). Another example of a genetic alteration that results in a defect in β-adrenergic signalling is the MLP knockout mouse that lacks the LIM-only protein of differentiated striated muscle cells (Arber, et al, 1997). Absence of this protein results in a phenotype of dilated cardiomyopathy with hypertrophy and failure with significantly impaired βAR signalling.

Summary of Reported Changes in the βAR Pathway

In summary, the results of studies of the βAR pathway in heart failure have some variability among the models, but in all cases, βAR signalling is impaired, and expression of the components of this pathway is in some way altered. In human heart failure and most animal models, β_1AR density and message is reduced whereas β_2 receptor density is largely unchanged. Coupling to adenylyl cyclase is impaired for both subtypes. Direct activation of adenylyl cyclase yields conflicting results: most human studies suggest that the cyclase moiety is intact, whereas many animal studies demonstrate that forskolin or manganese stimulated cyclase activity is depressed in failing hearts. Most studies report that βARK activity and protein is upregulated in heart failure, with no change in β-arrestin expression. An alteration in G-protein levels appears to be restricted to Gi: Gs levels are consistently reported to be unchanged, whereas Gi is reported to be upregulated in both human and some animal models of heart failure.

Transgenic Overexpression of Gαq in Murine Hearts

Cardiac specific overexpression of the α subunit of Gq in mice was achieved using the αMHC promoter. This animal was generated by the laboratory of Dr. Gerald Dorn (D'Angelo et al., 1997), prompted by the observation that hormones that activate receptors that couple to Gq (angiotensin II, endothelin 1, norepinephrine and prostaglandin F2α) stimulate cardiac hypertrophy. The transgenic line used in the majority of the current work has 40 copies of the transgene and 5 fold increased levels of Gαq protein compared to non-transgenic animals. This animal displays the characteristic physiological and biochemical markers of cardiac hypertrophy, including expression of the fetal gene program (ANF, α-skeletal actin and β-myosin heavy chain), intrinsic contractile impairment and chamber dilation, accompanied by a virtually absent response to infused catecholamines (Figure 3). Offspring from dual heterozygotes display a 50% mortality. In the Gαq mouse, pregnancy can evoke a post-partum cardiomyopathy.

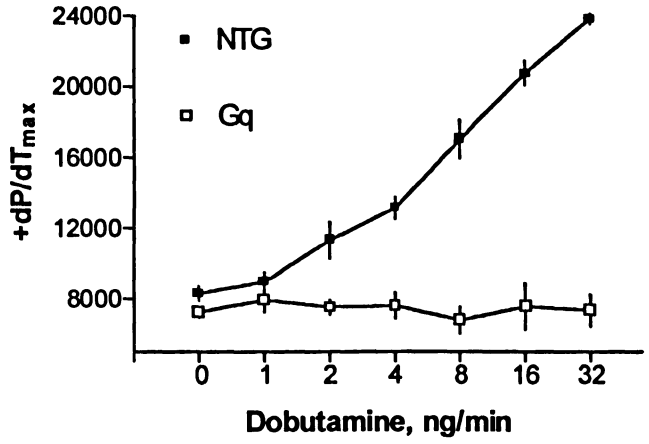


Figure 3: Physiological response to infused catecholamines in animals overexpressing Gαq compared to non-transgenic animals

In this work, specific hypotheses were tested regarding the consequences of Gαq mediated alterations of the βAR pathway. Specifically:

1. The Gαq animals exhibit cardiac hypertrophy and contractile dysfunction that is a result of defects of several components of the signalling pathway, including the receptor, G proteins, kinases, and adenylyl cyclase proteins.
2. Overexpression of the β_2AR in the Gαq mouse may partially restore βAR signalling by replacing the uncoupled pool of receptors. Overexpression of a βARK inhibitor in the Gαq background may partially restore function by decreasing βARK mediated receptor phosphorylation and uncoupling.
3. Adenylyl cyclase protein may be a limiting component of the βAR signalling cascade and overexpression of adenylyl cyclase would result in enhanced βAR signalling. To test this, adenylyl cyclase Type V was overexpressed in normal mice.
4. In the context of the Gαq mouse, decreased ACV protein limits signalling. To address this, ACV protein was overexpressed in the Gαq animals and receptor signalling was determined *in vitro* and *in vivo*.
5. The increased Gi protein observed in the Gαq mouse contributes to the depressed signalling of the βAR. This was explored *in vivo, in vitro*, and using a model cell based system.

We present here the results of extensive investigation of the

components of the βAR in the Gq model of hypertrophy and the effects of transgenic overexpression of components of the βAR pathway in the Gαq background.

Chapter 3

Materials and Methods

Adenylyl Cyclase Assay

The base of ventricles and septum (with the atria removed) were homogenized using a Brinkman Polytron in 5mM Tris (pH 7.4), 2mM EGTA (5/2) containing the protease inhibitors (5ug/ml) soybean trypsin inhibitor, leupeptin, apoprotinin and benzamidine, and IBMX (100μM). Homogenates was centrifuged at 500 x g for 10 minutes at 4°C. The pellet was discarded and the remaining supernatant was centrifuged at 19000 x g for 10 minutes. The pellet was resuspended in 5/2 buffer and $MgCl_2$ (final concentration 12 mM) was added. 20 uL aliquots were incubated with water (basal) or isoproterenol concentrations from 10^{-4} to 10^{-10} and a regeneration mix containing 2.8mM phosphoenolpyruvate, 0.06 mM GTP, 0.1mM cAMP, 0.12mM ATP, 4 units/mL myokinase, 10 units/mL pyruvate kinase and ^{32}P-αATP to a final reaction volume of 50 uL. Adenylyl cyclase activity was also determined by incubating membranes with 100uM Forskolin or 10mM NaF instead of isoproterenol. To estimate the contribution of β_1AR to the total βAR stimulation, other experiments were carried out by incubating membranes at 37 C for 5 minutes with 1.0 uM ICI118551 (a relatively selective β_2AR antagonist) to which isoproterenol was added (final concentration 10uM) and the incubations continued for 10 minutes. β_2AR mediated stimulation was estimated by carrying out reactions with the relatively selective partial β_2AR agonist zinterol under the same conditions.

Reactions were incubated at 37°C for 10 minutes before addition of cold stop solution containing excess ATP and cAMP, and ^{3}H-cAMP (25000cpm/mL) for determination of column recovery. Radiolabeled cAMP formed during the reaction was separated from ^{32}P-ATP by chromatography over alumina columns.

Bovine Rod Outer Segments (ROS) Phosphorylation Assay

Cytosolic βARK activity was measured by phosphorylation of rhodopsin derived from rod outer segments. Urea-treated ROS were prepared from dark-adapted calf retinas by stepwise sucrose gradient centrifugation. The ROS consisted of approximately 90% rhodopsin as assessed by Coomassie Blue staining and had no significant endogenous kinase activity. Hearts were homogenized in two mL of ice-cold buffer containing 25 mM Tris, pH 7.5, 5 mM EDTA, 5 mM EGTA and the protease inhibitors used for the cyclase activity determinations. The homogenates were centrifuged at 40,000 x g for 30 minutes. The pellet was then resuspended in the above buffer and homogenized.

For each phosphorylation reaction, 100 μg of proteins were incubated with 85 pmol of rhodopsin in buffer containing 0.1 mM ATP, 20 mM Tris, pH 7.4, 2 mM EDTA, 6 mM $MgCl_2$, and 10 μCi ^{32}PγATP. Incubations were carried out in the presence of light for 30 minutes at 30°C. Reactions were stopped by the addition of 50 μL SDS-PAGE loading dye (β-mercaptoethanol, glycerol, bromophenol blue). The total reaction was run on a 10% polyacrylamide gel and vacuum dried. The dried gel was then exposed

to film overnight.

Western Analysis

All western analysis was performed using 100 μg of protein homogenized in RIPA buffer. Protein was electrophoresed overnight on a 10% acrylamide gel. Proteins were then transferred to nitrocellulose at 50V for two hours. Nitrocellulose was washed at room temperature with a wash buffer of 200 mM NaCl and 50 mMTris for 15 minutes twice to remove gel stuck to membrane and the transfer solution. Membranes were blocked using 5% dried milk in wash buffer for 15 minutes. For βARK 1 analysis, membrane was probed for two hours with a 1:1000 dilution (in 5% dried milk in wash solution) of GRK2 antibody (Santa Cruz). The membrane was subsequently washed 2x for 15 minutes with wash buffer. The secondary antibody (goat-anti-rabbit conjugated to horseradish peroxidase) was diluted 1:6500 (5% dried milk in wash solution) and incubated for 1 hour. Secondary antibody was washed from membrane 2x for 15 minutes. The membrane was developed using enhanced chemiluminescence. Gs protein was detected using the Santa Cruz antibody at a 1:1000 dilution. Detection of Gi was done using AS/7 antibody (Calbiochem) at a 1:500 dilution. Detection of ACV was done using a 1:200 dilution of ACV/VI primary antibody from Santa Cruz. Secondary antibody and washes were performed as described above. For expression of phosphorylated phospholamban , immunoblots were carried out on isolated myocytes with and without isoproterenol treatment. (3uM for 5 minutes) Polyclonal antisera raised against a

phospholamban peptide phosphorylated at serine 16 (PS-16, PhosphoProtein Research) was utilized at a titer of 1:5000 as previously described (Luo, et al. 1998). PLB studies were done by the laboratory of Dr. E. Kranias by Raj Dash. Bands were quantitated using Imagequant software.

^{3}H Forskolin Binding

Freshly excised whole hearts were immediately homogenized in ice cold buffer containing 250mM sucrose, 1mM $MgCl_2$, 5 mM Tris pH 7.4 and the protease inhibitors leupeptin, benzamidine and trypsin inhibitor (all at 5ug/ul). The homogenate was filtered through nylon mesh and centrifuged at 40,000 x g for 10 minutes. The resulting pellet was resuspended in buffer containing 8mM $MgCl_2$, 0.08mM ascorbic acid, 50 mM HEPES, pH 7.4 and the protease inhibitors described above. Membranes were again filtered through nylon mesh. 200-300 ug of protein were incubated with 40nM ^{3}H-Forskolin with or without unlabeled forskolin in a final volume of 250 uL for 60 minutes at 25°C. Reactions were terminated by dilution with cold wash buffer (10mM Tris, pH 7.4) and vacuum filtration. Specific forskolin binding was determined by subtraction of nonspecific binding (in the presence of 10uM forskolin) from total binding (labeled forskolin only).

Southern Analysis

Southern analysis was used to identify the presence of the αMHC-ACV transgene and the αMHC-β_2AR. DNA was extracted from tail clips from three-week-old mice using potassium acetate precipitation. Ten

micrograms of DNA were digested with the restriction enzyme EcoRI (Gibco BRL) for detection of the ACV transgene, (Figure 15) and BglII (Gibco BRL) for detection of the β_2AR transgene. The entire reaction was loaded on a 0.8% agarose gel, and electrophoresed overnight. The next day, the gel was stained with ethidium bromide to visualize the digested DNA and photographed. The gel was washed with 0.25 M HCl to hydrolyze the digested DNA. The gel was then washed with a denaturation solution containing 0.5 M Tris and 1 M NaOH for 30 minutes, then a solution containing 1 M Tris and 1 M NaCl, pH 7, to neutralize the gel. The DNA was then transferred overnight to a nylon membrane. The membrane was subjected to both the denaturation solution above, then the neutralizing solution prior to UV cross-linking. For the ACV southern, the membrane was then hybridized overnight with a probe isolated from a EcoRI digest of the αMHC- ACV construct. This probe is a 3.9 kB fragment containing 1.9 Kb of the αMHC promoter region and the first 2.9 Kb of ACV. For the β_2AR southern, the membrane was hybridized with a 1.8 Kb fragment of the αMHC-β_2AR construct. This fragment, as the ACV probe, contains both αMHC and β_2AR sequence. After two washes with a wash solution containing 0.3 M NaCl/0.030 M Sodium Citrate/0.1%SDS (low stringency wash) and two washes with 0.03 M NaCl/0.0030 M Sodium Citrate/0.01%SDS (high stringency wash), the membrane was exposed to film overnight at room temperature.

Radioligand binding

Membranes from adenylyl cyclase experiments were used for radioligand binding. Membranes were incubated at room temperature in a total volume of 250 μL for two hours with 400 pM [^{125}I]CYP in the absence (Total) and presence (Non-specific) of a saturating concentration (1uM) of alprenolol. Assays were stopped by dilution with cold wash buffer (10 mM Tris, pH 7.4) and vacuum filtration through Whatmann glass fiber filters. Bound [^{125}I]CYP was counted using a γ counter. Specific binding was determined by subtraction of Nonspecific counts from Total counts. For agonist competition stuides, excised hearts were homogenized in 1 mL of 5 mM Tris/2 mM EDTA with the protease inhibitors benzamidine, leupeptin and trypsin inhibitor. The homogenate was then centrifuged at 40,000 x g for 10 minutes. The membranes were homogenized and centrifuged two more times, for a total of three high-speed spins. The final pellet was resuspended in a minimal volume of 50 mM Hepes/5 mM $MgCl_2$ pH 7.4. Approximately 150 ug of heart protein was incubated with 40pM of [^{125}I]CYP and a concentration of isoproterenol ranging from 10^{-10} to 10^{-3} M. The reaction was incubated at 37° C for one hour before terminating with cold wash buffer. Membranes were filtered and counted as in radioligand binding assay above. Data fitted to a sigmoidal dose response curve where appropriate and the goodness of fit and EC50 was determined using Prism GraphPad software.

Generation of Transgenic ACV Animals

Cardiac-specific expression of Adenylyl Cyclase Type V (ACV)

was achieved using the murine α-myosin heavy chain (αMHC) promoter that directs expression to the atria and ventricles of the heart (Subramaniam, et al 1991). cDNA for rat ACV was obtained from R. Premont, Duke University. A 4.5 Kb XhoI digest of this construct consisted of 434 bp of 5' ACV untranslated sequence, followed by the 3787 bp of ACV coding region sequence and 231 bp of 3' untranslated sequence. This fragment was ligated into the SalI site of the full length αMHC promoter. The sequence was confirmed by sequencing. DNA for microinjection was liberated from the αMHC-ACV plasmid by BamHI digestion. Microinjection into the male pronuclei if FVB/N embryos was carried out by standard procedure. Pups were screened via Southern analysis as described above. F1-F4 generation mice of 10-16 weeks of age were utilized, in parallel with age-matched nontransgenic littermates, for all studies.

Cell culture methods: Transient transfection of β_2AR and Gi and Pertussis Toxin Treatment

HEK293 cells at 40-50% confluency were transfected with 10 μg of β_2AR DNA in pBC and 5 μg of PRSVT with and without 10 μg of the α subunit of Gi2. Appropriate volumes of DNA were added to Dulbecco's Modified Eagles Media (DMEM) containing 0.1 mM chloroquine and 0.25 mg/mL DEAE/Dextran for one hour at 37°C at 5% CO_2. After aspiration of this solution, cells were incubated with 10% DMSO in Hank's Balanced Salt Solution (HBSS) for 2 minutes. Plates were washed with HBSS and incubated with DMEM until cells reached 100% confluency. 100 ng of

PTX/mL of media was incubated with 90% confluent HEK293 cells stably transfected with wild type β_2AR for 8 hours prior to treatment with agonist or vehicle. After eight hours of PTX treatment, 10 μM isoproterenol and 100 μM ascorbic acid or ascorbic acid alone was added to the cultures and incubated for one hour at 37°C. Cells were then prepared as described below for determination of adenylyl cyclase activity.

Adenylyl Cyclase Assay for HEK 293 cells

Cells were treated with vehicle or 10^{-5} M isoproterenol for 15 minutes at 37°C. After incubation, cells were washed 5x with cold phosphate buffered saline. Cells were scraped from flasks in buffer containing 5 mM Tris, pH 7.4 and 2 mM EDTA pH 8.0 and spun at 40,000 x g for 10 minutes. Cells were resuspended in a final volume of one ml of buffer containing 75 mM Tris,pH 7.4, 4 mM $MgCl_2$, and 2 mM EDTA, pH 8.0 and homogenized using a Brinkman polytron. 20 μL aliquots were incubated with water (basal) or isoproterenol concentrations from 10^{-4} to 10^{-10} M and a regeneration mix containing 2.8 mM phosphoenolpyruvate, 0.06 mM GTP, 0.1 mM cAMP, 0.12 mM ATP, 4 units/mL myokinase, 10 units/mL pyruvate kinase and ^{32}P-dATP to a final reaction volume of 50 μL. Maximal adenylyl cyclase activity was also determined by incubating with 100 μM Forskolin or 10 mM NaF instead of isoproterenol. In some cases, myocytes were isolated from the hearts and membranes prepared. Reactions were incubated at 37°C for 10 minutes before addition of cold stop solution containing excess ATP and

cAMP, and ^{3}H-cAMP (25,000 cpm/mL) for determination of column recovery. Radiolabeled cAMP formed during the reaction was separated from ^{32}P-ATP by chromatography over alumina columns. % Desensitization was calculated as 1-(maximal response- basal activity of isproterenol exposed cells)/(maximal response-basal response of unexposed cells) x 100.

Protein Kinase A Activity

PKA Activity was determined in preparations from whole hearts and in isolated myocytes. (Myocyte isolation protocol described below.) Whole hearts from transgenic or non-transgenic animals were placed in a 37°C bath of Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with or without 10 uM isoproterenol (final concentration) for five minutes to stimulate protein kinase A via the β-adrenergic signalling pathway. Hearts were then frozen in liquid nitrogen until assayed for PKA activity. Hearts were then homogenized in extraction buffer (50 mM Tris, pH 7.5, 5 mM EDTA) and centrifuged at 40,000 x g for 30 minutes. Aliquots of the resulting supernatant were then incubated in a reaction containing 50 uM Kemptide, 50mM Tris (pH 7.4), 10 mM $MgCl_2$, 100uM ^{32}P-γATP, 0.25 mg/ml bovine serum albumin, and either PKA inhibitor, cAMP (activator) or neither. The reaction was carried out at 30°C for 10 minutes. Reactions were terminated by spotting aliquots of the reaction on phosphocellulose discs. Discs were then washed with 1% phosphoric acid twice for five minutes and distilled water twice for five minutes before counting on a scintillation counter. Isolated myocytes were treated in the same manner as whole hearts, but were not frozen at any point

during the procedure. Briefly, myocytes were treated with 10uM isoproterenol or vehicle for 10 minutes at 37 °C. Cells were pelleted by centrifugation at 200xg for one minute and resuspended in a minimum volume of extraction buffer. Myocytes were then subjected to brief sonicatation to disrupt membranes, and then centrifuged at 40,000xg for 10 minutes. The supernantant was used to assess PKA activity in reactions identical to those described above.

PKC Expression/Function

All PKC data was obtained from the laboratory of Dr. Gerald Dorn.

Phospholipid-stimulated incorportation of ^{32}P into PHAS-I (d'Angelo et al. 1997) was utilized as an assay of total PKC activity in mouse hearts using components from Stratagene and Amersham. Briefly, mouse hearts frozen at −80°C were thawed, homogenized and separated into cytosolic and membrane fractions by centrifuagion at 100,000 x g x 30 minutes. 50 μg of each fraction was assayed for PKC activity by coincubation for 10 minutes at 30°C with 0.1 mM ATP plus 1μCi [^{32}P]ATP and 0.5 μg/μl PHAS-I in the presence and absence of phospholipid and $CaCl_2$. 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-PAGE gels and phosphorylation of the 21 kDA PHAS-I protein quantified using a PhosphorImager. Results are shown for whole homogenate (total PKC content) or membrane versus cytosolic activity (endogenous activation).

PKCα and PKCε mRNA were analyzed using a modification of a previously described PCR-based method which permits the simultaneous amplification of multiple PKC isoforms using degenerate oligonucleotide primers complementary to conserved sequences in cysteine rice 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 quanitated 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 PKCα and PKCε as radiolabled probes.

Patch Clamp Studies

All patch-clamp studies and myocyte isolation were performed by Dr. A. Yatani. Briefly, single ventricular myocytes were isolated from the hearts of non-transgenic and ACV or ACV/ Gαq mice and whole-cell currents were recorded using patch-clamp techniques as previously described. Briefly, the heart was perfused with Ca2+-free Tyrode's solution containing collagenase type I (Worthington; 0.5 mg/ml) and bovine serum albumin (1mg/ml) for 30-

40 minutes by the Langendorf method at 37°C. At the end of the perfusion period, the heart was removed and left ventricular tissues sieved though 200 um nylon mesh, and centrifuged for 2 minutes at 1000xg. Isolated cardiomyocytes were stored in low-Cl^-, high K^+ medium and all experiments were performed at 20-22°C. The patch pipettes had a resistance of 2 MΩ 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. 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 Cs 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 from other membrane currents such as Na^+ and K^+ channel currents and also Ca^{2+} flux through the Na^+/Ca^{2+} exchanger. Data are presented as mean ± SE of N number of myocytes studied.

Measurement of Cardiac Gene Expression

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 (Jones, et al. 1996). Briefly, two μg of RNA was denatured and blotted to Hybond N+ membranes. After blotting, membranes were

prehybrized for one hour at 55°C in a solution containing 5X SSC, 0.5% SDS, 1X Denhardts, and 90μg/ml salmon sperm DNA. Samples were then hybridized with ^{32}P labeled DNA probes for five hours 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. All dot blots for these studies were done by the laboratory of Dr. Gerald Dorn II, University of Cincinnati.

Histology

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

Echocardiography

All echocardiography data was obtained by Dr. Gerald Dorn, II. Noninvasive cardiac function was assessed using two dimensional guided M-mode echocardiography. Mice were lightly anesthetized using Avertin prior to analysis. Left ventricular end systolic dimensions (ESD), end diastolic dimensions (EDD), and septal and posterior wall thickness were recorded on an ATL HDI 3000 digital echocardiography system using a linear array CD-5 transducer. Fractional shortening was calculated as (EDD-ESD)/EDD. Left Ventricular Mass (LVM) was calculated as $[(LVEDD+SWT+PWT)^3$ –

$LVEDD^3$] x 1.832 (D'Angelo, et al. 1997), where LVEDD is left ventricular end diastolic dimension, SWT is septal wall thickness, and PWT is posterior wall thickness.

In vivo Hemodynamics

All *in vivo* hemodynamic studies were done by the laboratory of Dr. John Lorentz. Closed chest hemodynamics were performed on 12-16 week old mice sedated with ketamine/thiobutabarbital anesthesia as previously described (Turki, et al. 1996).

Statistical Analysis

All curve fits, means and standard errors were generated using Prism GraphPad software. Analyses of Western blots were done using OFOTO scanning software and ScanAnalysis software. Probability (P) values were determined using an unpaired Student's t-test (assuming equal variance) utilizing GraphPad Instat software. Significance was assigned for P values $\leq$ .05. All data is reported as mean $\pm$ standard error of the mean.

Chapter 4

Results: Determination of βAR Signalling Defects in the Gαq Mouse

The first hypothesis tested was that the impaired contractile responses to β-agonists observed in the Gαq mouse was a consequence of defects in the βAR signalling pathway, potentially including G proteins, kinases, adenylyl cyclase and the receptor itself. Receptor density, affinity and receptor-Gs coupling were assessed using radioligand binding studies in the Gαq mouse. To determine defects occuring downstream of the receptor, adenylyl cyclase activity in response to sodium fluoride (which activates G proteins), forskolin and manganese (which directly activates adenylyl cyclase) were also utilized. None of these, however, are adequate by themselves to delineate a specific defect. Thus, in order to identify mechanism (for example, decreased protein synthesis vs. decreased function), functional studies and protein expression were examined in tandem where possible. Assessment of protein levels of βARK, G proteins and adenylyl cyclase were performed using the Western blotting technique. G protein receptor kinase (GRK) activity was assessed using the bovine rod outer segment assay, which determines the ability of GRK to phosphorylate purified, light activated rhodopsin. This assay is limited, however, in that it cannot distinguish among the different GRK isoforms, and does not provide any information on changes in activity of individual GRK isoforms. The results of these studies were used to assess how various proteins in the βAR pathway can be altered as a result of Gq induced hypertrophy. Although the Gq mouse is only a model of

hypertrophy and contractile dysfunction, these studies investigate mechanisms whereby βAR signalling becomes impaired in hypertrophy and ventricular failure, and allows delineation of specific pathological mechanisms that may be targeted in the context of human cardiac dysfunction.

Previous work by D'Angelo, et al. 1997, characterized the physiology of transgenic mice overexpressing the alpha subunit of Gq. Gαq transgenic animals display markedly depressed contractile responsiveness to infused β-agonists. Figure 3 (see Background) depicts the depressed inotropic response to infused agonist in the Gαq mouse compared to non-transgenic animals. Heart rate was also significantly depressed in Gαq animals compared to non-transgenic controls. Pacing of Gαq hearts to non-transgenic rates did not restore basal +dP/dt or dobutamine stimulated contractility.

To begin to understand the underlying molecular mechanisms of contractile impairment as a result of Gq overexpression, studies were carried out investigating receptor function and proteins downstream of the receptor. As many models of heart failure are characterized by a downregulation of total β-adrenergic receptors at the membrane, radioligand binding studies of membranes from control and Gαq hearts were performed to determine if receptor number was altered in transgenic hearts. These experiments showed no significant change ($p = 0.2$) in total β-adrenergic receptor density: Gαq

animals expressed 47.2±9.8 fmol/mg protein and control animals expressed 35.5±3.8 fmol/mg protein (Figure 4).

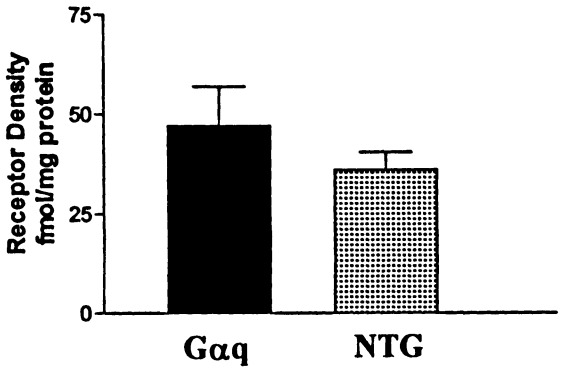


Figure 4: Radioligand binding of non-transgenic (NTG) and Gαq animals. *No significant difference was observed in total receptor number in Gαq animals compared to non-transgenic, n=4-5.*

Although receptor number was not changed in the transgenic Gαq mouse, it is possible that the receptor is post-translationally regulated via phosphorylation, given the observation that various isoforms of PKC are upregulated in the Gαq mouse (D'Angelo et al., 1997; Dorn et al. 1999). Alternatively, changes in G proteins could affect the coupling of the receptor to Gs, altering the relative ratio of high affinity receptors formed through receptor-Gs interaction. To address the possibility that the high affinity binding sites of the βAR might be altered, competition binding curves (Figure 4) were performed using ^{125}I-cyanopindolol and isoproterenol in the absence of guanine nucleotides, which allows the accumulation of the agonist high affinity receptor complex. Data from such studies generate curves with a biphasic nature, providing for calculation of the affinity and proportion of receptor in the high (K_H) and low (K_L) affinity states. The first component of

the curve represents receptors in the high affinity state, and the second phase of the curve represents the population of low affinity receptors. In the Gαq mouse, the proportion of receptors in the high affinity conformation was markedly reduced, from 59.1±8% in non-transgenic animals to 27±3% of the total receptor population in Gαq animals ($p < 0.01$; Table 1 and Figure 5). The K_H and K_L did not differ, although the ratio of the two affinities (K_L/K_H, a measure of the potential transfer of free energy upon activation) was decreased ~50% in the Gαq mice.

Table 1

Results from competition analysis of Gαq and Non-transgenic (NTG) Cardiac Membranes in the absence of GTP. Increasing concentrations of isoproterenol (100pM to 100uM) were incubated with membranes and 40nM [^{125}I]CYP. See Figure 5. $\%R_H$ refers to the percent of receptors in high affinity confomation, whereas $\%R_L$ represents the percentage of receptors in the low affinity conformation. (* $P<0.01$)

	K_H (M)	K_L (M)	$\%R_H$	$\%R_L$	K_L/K_H
NTG	$1.6\pm0.7\times10^{-9}$	$2.7\pm1.4\times10^{-7}$	62 ±10%	27.2±3%	168
Gαq	$1.1\pm0.1\times10^{-9}$	$7.5\pm0.9\times10^{-8}$	29±4%*	59.1±8%*	71

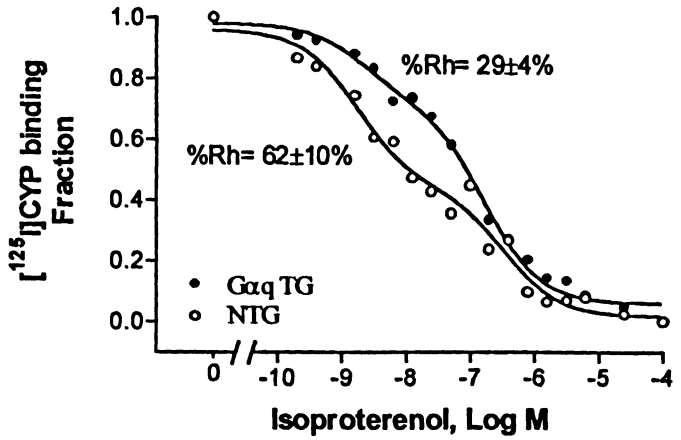


Figure 5: Competition Binding of Gαq Cardiac Membranes and Non-transgenic (NTG) Membranes in the Absence of GTP

To more fully understand the role of downstream components contributing to the Gαq phenotype, basal and agonist stimulated activity of the βAR effector protein, adenylyl cyclase, were measured *in vitro*. Interestingly, both basal (21.8±2.7 vs. 44.6±4.5 pmol cAMP/min/mg protein, $p < 0.005$) and agonist stimulated (40.8±3.1 vs. 85.9±4.2 pmol cAMP/min/mg protein, $p = 0.0001$) AC activity was markedly depressed, despite no change in receptor number. No change in the EC_{50} of the isoproterenol dose-response curve was observed in Gαq animals. (Log EC_{50} = -6.7±0.3 in control animals vs. -6.5±0.4 in Gαq animals, $p = 0.74$. Figure 6.)

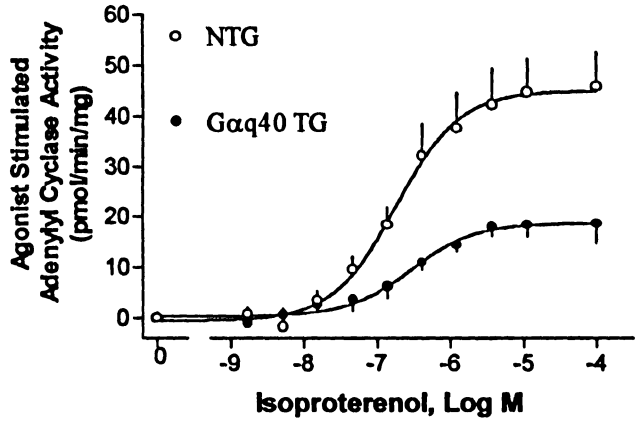


Figure 6: Adenylyl Cyclase activity in response to isoproterenol in non-transgenic (NTG) and Gαq Cardiac Membranes. *Both basal and maximally stimulated adenylyl cyclase activity are depressed in the Gaq mouse, n=4, p<0.005. Basal values are subtracted for clarity.*

To assess βAR subtype-specific coupling, adenylyl cyclase activities were determined with the relatively β_2AR selective partial agonist zinterol, and with isoproterenol in the presence of the relatively selective β_2AR antagonist ICI118551 for an indication of β_1AR coupling. While this

approach does not provide for absolute selective activation of one or the other subtype, comparisons between Gαq and NTG mice do allow for a relative determination of potential differences in signalling under identical conditions. In four such experiments, the zinterol response over basal was 11.3±3.6 pmol/min/mg for NTG compared to Gαq mice, equivalent to a ~40% desensitization of the β_2AR. β_1AR stimulation, assessed as described, was 10.1±2.3 pmol/min/mg over basal with NTG mice, compared to only 2.0±1.0 pmol/min/mg with the Gαq mice ($p<0.02$). Thus, β_1AR function was impaired ~80% in the Gαq mice.

Taken together, these studies reveal a receptor-G protein coupling defect in the Gαq mice. Such an alteration of high affinity receptor populations can be the result of phosphorylation of the receptor, a decrease in the available amount of Gs, or an increase in low affinity coupling of the receptor to other G proteins, such as Gi. To better understand which mechanisms may be contributing to the uncoupling of the receptor, Western blots were carried out to determine G protein levels, and activity assays were performed to determine the role of PKA, PKC and GRKs.

Western analysis of non-transgenic and Gαq animals showed no difference in Gs protein levels (Dorn et al. 1999). Giα_2 and Giα_3 levels, however, were upregulated approximately three-fold in the Gαq mouse, as detected by Western analysis. (Dorn et al. 1999.) To assess the possibility

that increased levels of Gi could affect receptor signaling, a model cell-based system was utilized. (See Chapter 8).

The potential role of increased levels of kinases known to phosphorylate the β_2AR was examined. As discussed, human failing hearts and many models of hypertrophy demonstrate increased levels of GRK2 (βARK1) and GRK activity. Western analysis of GRK2 in the Gαq mouse, however, showed a significant downregulation of protein in the transgenic animals compared to control (77227±2679 activity units by densiometric analysis in Gαq animals vs. 98426± 1785 in non-transgenic animals; P = 0.0006; Figure 7).

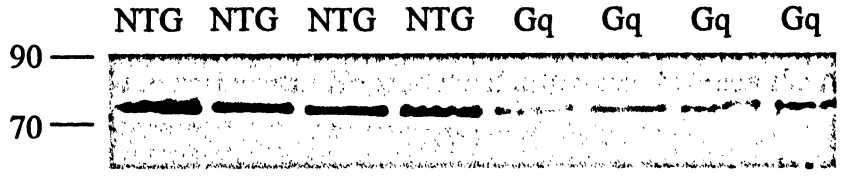


Figure 7: Western Analysis of GRK2 (βARK 1) in Gαq and non-transgenic (NTG) Animals. *GRK2 levels were found to be significantly down in the Gαq animals. n=4, p< 0.001.*

Western analysis provides an indication of total protein but does not address changes in activity. To determine the activities of total GRK in Gq and non-transgenic hearts, cytosolic extracts were prepared and incubated with ^{32}P-γATP and isolated rod outer segments (ROS) from bovine retinas. Total GRK activity is then measured as phosphoryation of rhodopsin (a G protein coupled receptor in ROS). This assay is limited in that it measures

activities of all GRKs, and cannot distinguish among the different isoforms. Analysis of total GRK activity, using this assay, showed no change in activity (6758±229 counts in Gαq animals vs. 8962±1457 counts in non-transgenic animals, p = 0.2), although there was a trend toward decreased activity in Gαq mice (Figure 8).

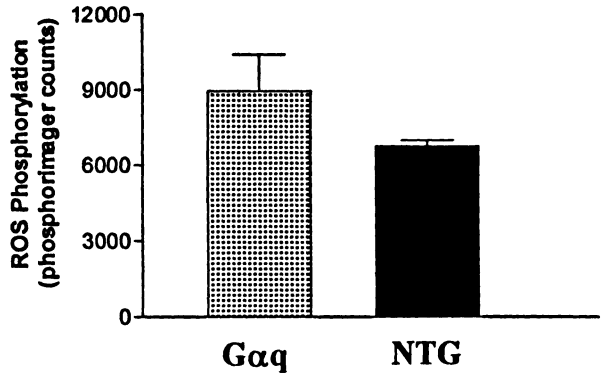


Figure 8: ROS Assay: GRK Activity of Gαq and Control Animals. *Phosphorylation of rhodopsin, a GRK substrate, was used to determine total GRK activity in non-transgenic and Gaq cardiac homogenates. Total GRK activity was unchanged in transgenic animals compared to controls. Shown is a mean of 4 experiments. No statistical difference between the two groups was observed.*

As GRK levels are clearly not *increased* in the Gαq animals, it is unlikely that GRK mediated phosphorylation plays a role in uncoupling of the receptor. PKA, however, regulates β-adrenergic receptors via phosphorylation, and could contribute to the depressed responsiveness to β-agonists. Since basal adenylyl cyclase activity was not increased in transgenic animals, PKA levels would not be expected to be higher in Gq animals. Indeed, in transgenic cytosolic preparations, basal or maximally stimulated PKA activity was unchanged compared to non-transgenic. (See Figure 9.)

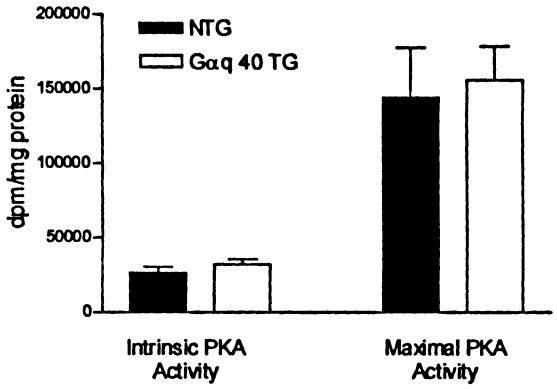


Figure 9: PKA activity in non-transgenic and Gαq homogenates. *Homogenates from Gaq and non-trangenic hearts were used to phosphorylate a synthetic PKA substrate in the absence (intrinsic) and presence (maximal) of cAMP. No differences were observed. Shown is a mean of 4 experiments.*

PKC activity was investigated as a potential contributor to receptor desensitization and uncoupling. All PKC data were obtained from the laboratory of Dr. Gerald Dorn. Previous studies of the Gq mouse (D'Angelo, et al., 1997) showed enhanced translocation of PKCε. Total PKC in homogenates from Gαq mice (measured as phosphorylation of PHAS-1 protein by whole heart homogenates) was found to be increased 2.6±0.8 fold over non-transgenic, likely due to sustained diacylglycerol activation by Gαq. The ratio of activated PKC to cyosolic (inactive) PKC was not different between the two groups. Thus, absolute activated PKC is higher in Gαq hearts due to the increase in total PKC levels (figure 10, Dorn et al. 1999). To determine which PKC isoforms undergo changes in expression, PKC isoform content was assayed by quantitative immunoblotting. The most abundant PKC isoform was PKCα which was expressed at levels

approximately 5 times that of PKCε. Ventricular PKCα in Gq overexpressors was upregulated by 76% (NTG 712±114 vs Gq 1255±158 ng/mg protein, n=4 pairs, $p<0.01$), whereas PKCε was downregulated in Gαq mice by 26% (NTG 158±10 and Gq 117±12 ng/mg protein, n=8 pairs $p<0.02$). PKCδ and η did not differ. Intrinsic activation of PKCα and ε isoforms was determined by their relative particulate and soluble partitioning. Consistent with the results of the phophorylation studies, the absolute amount of particulate-associated PKCα in Gq mice was greater than NTG, indicating an increase in the amount of activated PKCα in Gq mice, although the ratio of particulate to cytosol PKCα was not changed in the Gq mice (NTG0.51±0.13 vs Gq 0.47±0.14, n=4, p=NS). Confirming data presented previously (D'Angelo et al 1997), PKCε translocation was significantly increased (NTG 1.17±0.09 vs Gq 2.38±0.18, n=8 pairs, $p<0.001$; Dorn et al. 1999).

Studies using RT-PCR and Northern blots of PKC isoform mRNA from nontransgenic and Gq mouse hearts indicate more abundant PKCα mRNA by ~2 fold in the Gαq overexpressors, consistent with the observed increase in PKCα protein. In contrast, Gαq PKCε mRNA levels are identical to controls. Thus, these studies suggest that upregulation of PKCα in Gαq overexpressors may be transcriptionally mediated, but that downregulation of PKCε is post-transcriptional (Dorn et al. 1999).

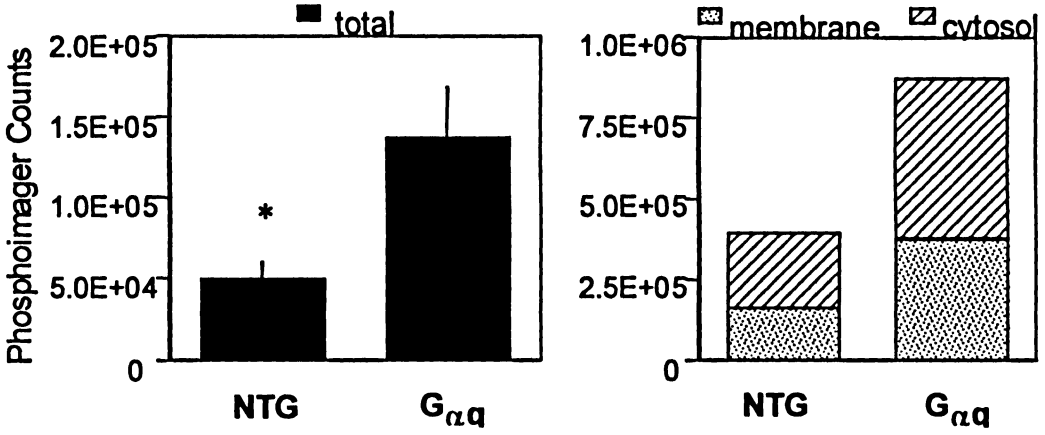


Figure 10. ***Phosphorylation of a synthetic PKC peptide substrate by homogenate, cytosolic, and membrane fractions from hearts.*** *The total PKC activity is increased in the Gαq mice, and the ratio of cytosolic to membrane fractions is maintained. Shown are results of four to six independent experiments. Experiments shown in this figure were performed by the laboratory of Dr. Gerald Dorn II. (Figure from Dorn, et al. 1999). *=p<0.001.*

A defect in agonist mediated AC activity could also be the result of a defect of downstream components of the βAR pathway. Forskolin, a direct activator of adenylyl cyclase, was used to determine the maximal activity. Adenylyl cyclase activity in response to forskolin was 283.5±61.2 pmol cAMP/min/mg protein in Gαq animals vs. 519.3±54.2 in control animals (P=0.02), representing a 46% decrease (Figure 11). Thus, one component of the impaired signalling is likely a result of the significantly depressed activity of the adenylyl cyclase moiety. A change in the absolute or relative concentrations of the G proteins Gs and Gi could influence signalling, either by modifying receptor coupling, or via a tonic effect on adenylyl cyclase activity. Sodium fluoride stimulated adenylyl cyclase activities were also decreased in Gαq animals (58.8±7.2 vs. 147.1±20.0 pmol cAMP/min/mg

protein in control animals, p=0.016, figure 6), representing more than a 50% decrease in this response. This depressed responsiveness to sodium fluoride could reflect changes in Gs and/or Gi, or, could also be the consequence of impaired adenylyl cyclase protein.

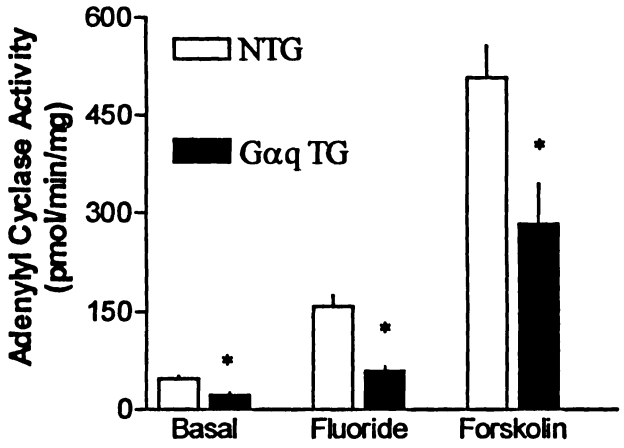


Figure 11: Adenylyl Cyclase Activity in response to Forskolin, 100uM (panel A) and Sodium Fluoride, 10 mM (panel B) in cardiac membrane preparations from non-transgenic (NTG) and Gαq animals. *Shown are the results from 4 separate experiment,* *= $p \leq 0.02$.*

The observed decrease in sodium fluoride and forskolin stimulated adenylyl cyclase activity could in part be due to the observed increase in Gi. To assess the potential contribution of the increase in Gi to the phenotype, mice were treated *in vivo* with pertussis toxin (100ug/kg) which dissociates receptor-Gi interaction by ADP-ribosylation of the αi subunit. However, three of the four Gαq mice treated did not survive, whereas the non-transgenic animals treated with toxin suffered no untoward effects. Therefore, studies with pertussis toxin were limited to isolated myocyte

studies. Myocytes were isolated and treated with 5ug/ml toxin for six hours. Basal and isoproterenol stimulated adenylyl cyclase activities were then determined in membranes preparations from treated and untreated myocytes. In non-transgenic mice, both basal and isoproterenol stimulated activities were increased with toxin treatment, but the fold stimulation was unchanged. In membranes from Gαq mice, basal and agonist stimulated activities were increased with pertussis toxin treatment, but the fold stimulation was also increased, from 2.29±0.51 to 3.38±0.23 ($p<0.05$). The fold stimulation with treatment was still less than that observed in non-transgenic animals (4.03±0.71), suggesting that factors beyond Gi upregulation contribute to the overall signalling defect. The observed decreases in forskolin and sodium fluoride responsiveness were unchanged with toxin treatment (see Figure 12).

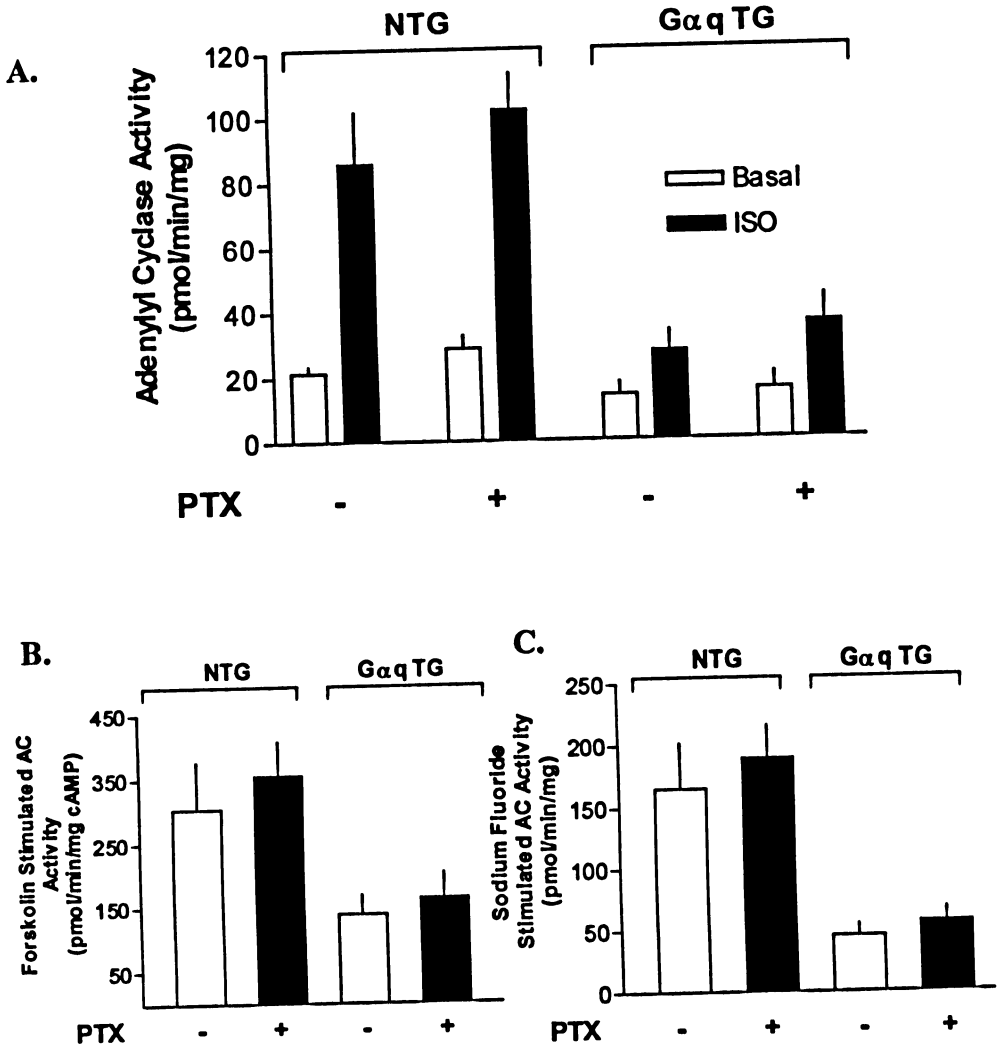


Figure 12. ***Pertussis toxin treatment of Non-transgenic and Gαq myocytes.*** *Intact myocytes were isolated and treated with 5ug/ml pertussis toxin or vehicle alone for 6 hours, membranes prepared, and adenylyl cyclase activites determined in the presence of water (basal), 10 uM isoproterenol (ISO) (Panel A), 10 mM NaF (Panel C), or 100 uM forskolin (Panel B). Shown are results from four experiments. PTX treatment improved adenylyl cyclase responsiveness in Gaq membranes but did not restore to non-transgenic values. (See text.) Forskolin and sodium fluoride stimulated responsiveness was not significantly improved with PTX treatment.*

The significantly depressed forskolin and sodium fluoride responsiveness in Gq membranes suggested a defect intrinsic to adenylyl cyclase protein. Possible alterations could include downregulation of AC protein or a post-translational modification via phosphorylation. To determine if AC protein was indeed altered in Gq mouse, Western blots and ^{3}H-forskolin binding assays were performed. Western analysis using a polyclonal antibody recognizing AC type V and VI suggested a possible isoform specific downregulation of type V adenylyl cyclase, as distinguished by molecular weight (Figure 13). To more accurately determine levels of AC protein, ^{3}H-forskolin binding was carried out. This method detected an approximate 50% reduction in total AC protein in Gαq cardiac membrane preparations (72±11 vs. 133±8 fmol/mg in non-transgenic animals, n=4, $p<0.01$, see Figure 14).

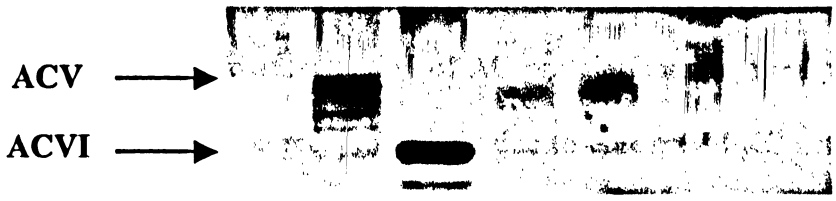


Figure 13: ***ACV downregulation in Gαq mice as determined by Western Analysis.*** *The decrease in ACV protein in Gaq animals was 32% compared to non-transgenic.*

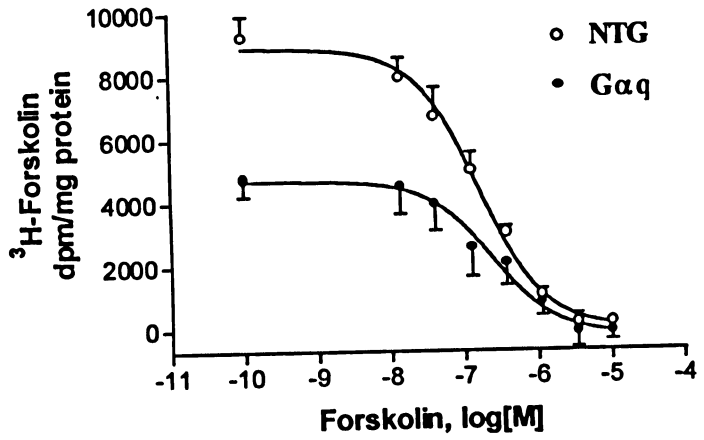


Figure 14: ^{3}H Forskolin Binding in Non-transgenic and Gαq Transgenic Animals. *^{3}H forskolin binding, used to quantitate changes in adenylyl cyclase expression was 46% less in Gαq transgenic mice compared to non-transgenic littermates (72±11 vs. 133±8, respectively, $p<0.01$).*

In summary, three major defects were identified which likely contribute to the impaired β-agonist mediated contractile response. These defects include a downregulation of AC protein/function, upregulation of Gi, and uncoupling of the receptor, likely due to enhanced PKC activity. Figure 15 depicts the three primary defects observed in the Gαq mouse and the subsequent approaches used to delineate the contribution of each of these defects to the overall phenotype. Specifically, the contribution of receptor uncoupling was addressed via transgenic overexpression of the β_2AR and a βARK-inhibitor (a 300 amino acid peptide which preferentially binds the βγ subunits that function to translocate βARK to the membrane; Koch, et al 1995). This was accomplished via mating of heterozygous β_2AR or βARK-

inhibitor expressing mice with the heterozygous Gαq animals. Overexpression of the receptor replaces the pool of receptors that have been functionally uncoupled from downstream effectors, thereby allowing assessment of the contribution of βAR uncoupling. Expression of the βARK inhibitor, likewise, would presumably relieve some receptor uncoupling resulting from βARK mediated phophorylation of the receptor, although the effects would be anticipated to be minimal, as GRK activity and βARK protein is not increased in Gαq animals.

The second major defect observed in Gαq animals was an increase in Gi levels on the order of approximately three fold. The possibility exists that such upregulation could result in an imbalance of G proteins that could influence tonic regulation of adenylyl cyclase or βAR signalling. Evidence for Gi coupling to the β_2AR has accumulated over the last several years, a phenomenon that has potential implications in the Gαq model. To assess the contribution of increased Gi, several distinct approaches were utilized. First, Gi coupling *in vivo* in the Gαq animal was investigated via intraperitineal injections of pertussis toxin (PTX), inactivating cellular Gi. Second, *in vitro* treatment of isolated myocytes from Gαq and non-transgenic animals with PTX was used to determine the consequences of Gi upregulation on βAR signalling. Finally, a cell based system using HEK-293 cells that allowed the manipulation of the ratio of receptor to Gi protein was used. This system was used to define the possibility of Gi coupling to the β_2AR under both

unstimulated and desensitized conditions.

The final series of experiments were done to address the role of adenylyl cyclase protein in βAR signalling in both normal physiological conditions and within the context of the Gαq mouse. As discussed, total adenylyl cyclase protein was found to be decreased by approximately 50% in the Gαq mouse, presumably due to an isoform specific downregulation of Type V AC. Work by Gao, et al and Roth et al suggests that adenylyl cyclase protein sets a limit on βAR signalling under normal conditions. This work, however, was done with the ACVI isoform, which is not the predominant human cardiac isoform. To determine potential for AC type V to limit signalling, transgenic mice overexpressing this isoform were generated and βAR function was assessed. These animals were then mated to the Gαq animals to replace downregulated AC protein, and the effects of restoration of this component of the pathway were determined.

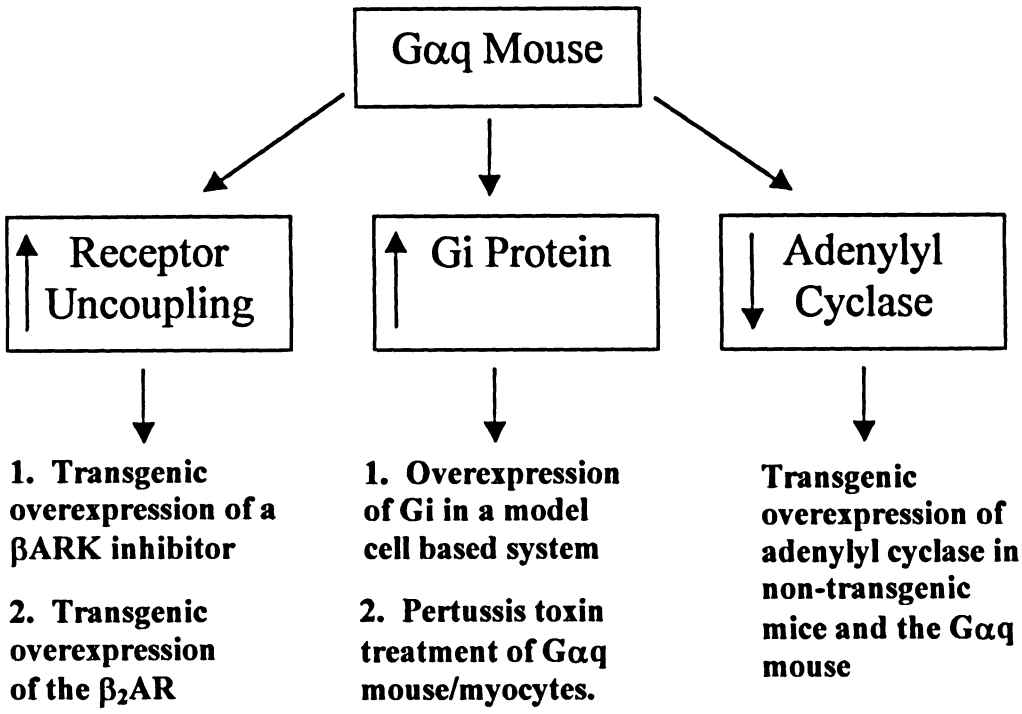


Figure 15: ***Diagram of primary defects observed in the Gαq mouse and subsequent approaches used to determine the contribution of the observed defect to the overall Gαq phenotype.***

Chapter 5

Results: β_2AR Overexpression in the Gαq Background

To better understand the contribution of decreased βAR coupling induced by Gαq overexpression, the Gαq overexpressing transgenic mouse line was crossed with pre-existing transgenic mice overexpressing the dominant-negative βARK (Koch et al., 1995) or the β_2 adrenergic receptor (Milano et al., 1994; Turki et al., 1996). These experiments were designed to identify the relative contribution of two specific proteins involved in hypertrophy and failure in this model. *In vivo* cardiac function and receptor coupling to adenylyl cyclase with progression to failure was assessed. These types of experiments fall into two categories. First, to rescue function by reversing an alteration in a signalling component (such as counteracting the effects of increased receptor phosphorylation with the βARK dominant negative), which addresses the contribution of downstream regulatory mechanisms. The second approach was to restore βAR signalling by "brute force" overexpression of the β_2 adrenergic receptor itself. Overexpression of receptor hypothetically allows replacement of the high affinity receptor population. Thus, these studies attempt to correct the receptor defect, leaving other potential mechanisms intact, allowing an assignment of the specfic contribution of this alteration on the Gαq phenotype.

To determine the effects of variable expression of the β_2AR in the Gαq background, transgenic mice were generated overexpressing the β_2AR

gene driven by the αMHC promoter. Mice positive for the transgene were identified by Southern analysis. The probe used to detect the transgene spanned the αMHC-β_2AR junction, and identified a discrete band of approximately 1.8 Kb. A representative southern blot for the β_2AR transgene is shown in Figure 16.

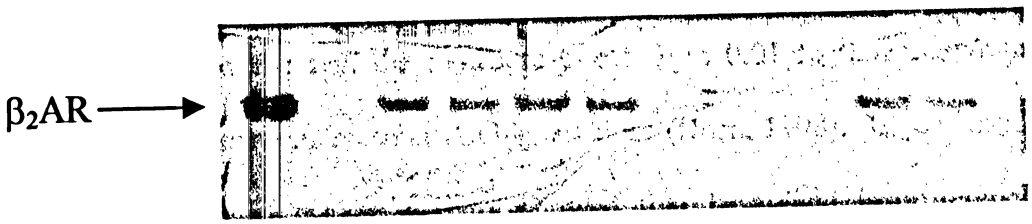


Figure 16: Representative southern blot identifying β_2AR transgene positive animals

Cardiac βAR density was determined for several lines of the β_2AR overexpressing mice that were crossed with the Gαq animals. The receptor density of the moderately expressing line (designated "Gq/β_2AR_M") was determined to be 3149±1026 fmol/mg protein, approximately 140 fold that of nontransgenic. The highest expressing line, designated "Gq/β_2AR_H", was determined to be 23,294±2438 fmol/mg protein, nearly 1000-fold nontransgenic levels. Additionally, the lowest expressing line, obtained from the laboratory of Dr. R. Lefkowitz, (Duke University, Durham, N. Carolina), was crossed with the Gαq animals and designated "Gq/β_2AR_L". This line was determined to have a receptor density 808±75 fmol/mg protein (30-fold overexpression) when crossed with the Gαq animals.

The lowest expressing line, Gq/β_2AR_L, displayed improvement in fractional shortening compared to the Gαq mouse (44±2 % vs. 32±2 %, p = 0.0054; NTG: 50±1%) and LV mass (74±1 mg vs. 90±3 mg, p = 0.0023; NTG: 65±4 mg). (All echocardiograph data was obtained by the laboratory of Dr. Gerald Dorn II, Cincinnati, OH). Additionally, basal left ventricular dP/dt max was improved (5592±653 mmHg/sec vs. 4557±468 mmHg/sec). However, intravenous administration of isoproterenol demonstrated no significant improvement in the inotropic effect from 0.01 to 0.32 ng/g/min and only a very small increase at the highest dose (Dorn, 1998). Biochemical analysis of adenylyl cyclase activity showed that basal and isoproterenol stimulated activity in the Gq/β_2AR_L animals was no different than that of Gαq mice (Figure 17). To determine if higher levels of β_2AR could improve function further, the β_2AR_M mouse (expressing 140x non-transgenic receptor levels) was mated to the Gαq mouse, anticipating improved biochemical and physiological rescue. Improvement of βAR signalling was observed (Figure 17), but physiological parameters were worsened. In this animal, higher levels of expression resulted in massive enlargement of the heart, and hypertrophy gene expression remained high. Additionally, expression of the βAR at these levels resulted in fibrosis not observed in Gq/β_2AR_L animals or Gαq animals. The highest expressing line, β_2AR_H, was concurrently crossed with the Gαq animals. However Gαq animals crossed with the highest expressing line of β_2AR did not survive past the age of five weeks and most of these animals died suddenly at three weeks with massively enlarged hearts.

Expression of the βARK mini-gene did not improve function of the Gαq animals. Left ventricular mass was unchanged with expression of the βARK inhibitor, and fractional shortening was not improved. (See Table 2). This is consistent with the observation that βARK is not upregulated in Gαq animals.

Table 2.

Fractional Shortening and Left Ventricular Mass in non-transgenic, Gαq and Gαq mice expressing the βARK inhibitor mini-gene. Cardiac function was not restored in Gαq mice, nor was hypertrophy reversed by βARK inhibition. (n=3-5 animals, $p > 0.05$).

	Non-transgenic	Gαq	Gαq + βARK mini-gene
Fractional Shortening	50 ±1	32 ± 2	27±1
LVM	65 ± 4	90 ± 3	91 ± 4

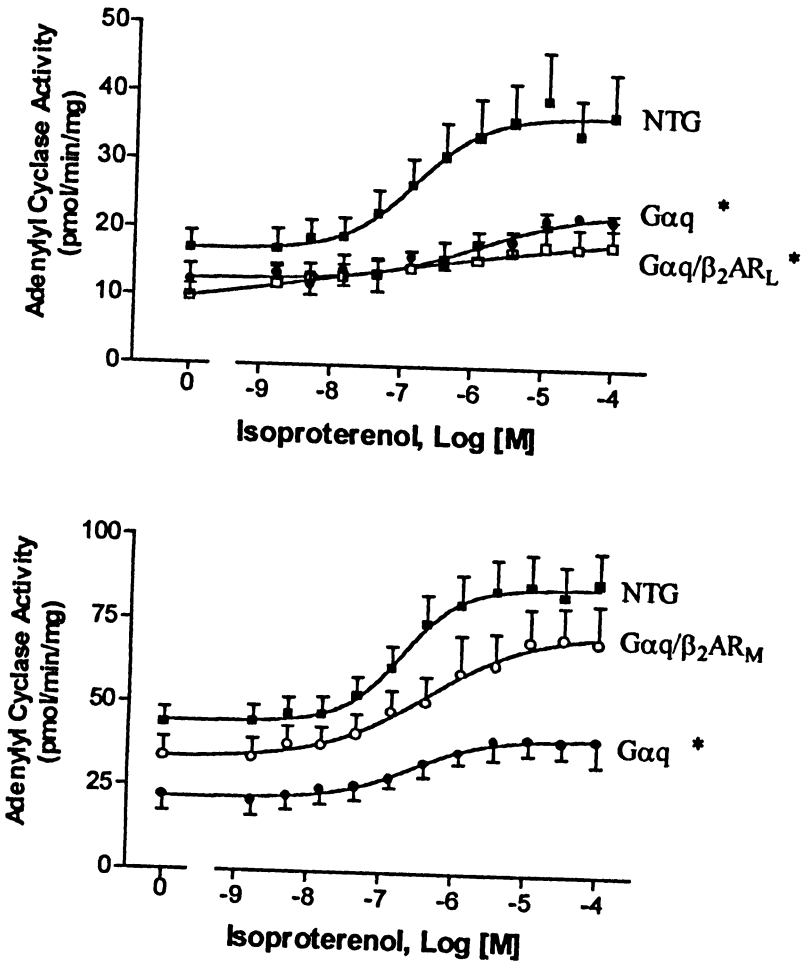


Figure 17: Adenylyl Cyclase Activity in response to Isoproterenol of cardiac membrane preparations from Gαq, Gq/β2AR (Low, Gq/β2ARL, and Moderate, Gq/β2ARM), transgenic mice, and non-transgenic littermates. *Adenylyl cyclase activity was not improved in Gαq animals with low levels of β2AR overexpression (60 fold), whereas signalling was markedly improved with moderate levels of overexpression (140 fold). (n=4-5). *, overall response different than non-transgenic, p<0.01.*

Chapter 6

Results: Adenylyl Cyclase Type V Overexpression

The relative stoichiometries of receptor, Gs and adenylyl cyclase have been reported to be 1:200:3. This finding has led to the theory that AC may be the limiting component of receptor signalling in the context of normal cardiac physiology. Since we have found that type V adenylyl cyclasse expression/function is reduced in the Gαq mouse and that this is likely a main component of the dysfunctional βAR signalling, transgenic mice were created overexpressing adenylyl cyclase type V (ACV) approximately 150% that of non-transgenic animals. This was accomplished using the αMHC promoter to drive expression in the atria and ventricles. With modest overexpression of ACV, enhanced receptor signalling might be expected if this protein does indeed limit the ability of receptor to signal to the interior of the cell. This model is also ideal for determining the effects of enhanced basal activity of adenylyl cyclase on downstream molecular components and overall cardiac physiology. Both biochemical and physiological consequences of AC overexpression were investigated using this model, including βAR mediated adenylyl cyclase responsiveness, PKA activity, *in vivo* hemodynamics and tissue histology.

Cardiac-specific expression of Adenylyl Cyclase Type V (ACV) was achieved using the murine α-myosin heavy chain (αMHC) promoter that directs expression to the atria and ventricles of the heart. Pups were screened

for presence of the transgene via Southern analysis using EcoRI digested genomic DNA extracted from tail clips as described in the methods. The ACV fragment used for microinjection consisted of the αMHC promoter, the ACV transgene, and the HGH/SV40 polyA tail. Figure 18 shows the αMHC/ACV construct used for microinjection.

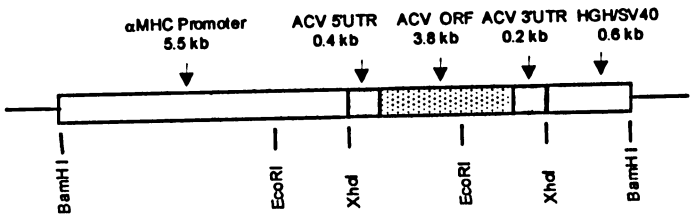


Figure 18: Adenylyl Cyclase Type V Construct

Initially, two lines of ACV positive animals were established, designated ACV-15.1 and ACV-14.3. For the majority of the experiments, however, only the 14.3 line was used. (Where not indicated, the 14.3 line is used.) Lines 15.1 and 14.3 carried approximately 80 and 400 copies of the transgene, respectively.

Animals carrying the ACV transgene have normal lifespans up to 10 months of observation and litter sizes are normal. Although ACV animals tended to be slightly smaller than non-transgenic animals (Table 3), heart to body weight ratios were not different, and there was no observable cardiac hypertrophy. Histological staining using Masson's trichrome stain, which

stains for collagen, and hematoxylin and eosin stain, show no pathological consequences of the transgene (See Figure 27). Lung and liver weights (indicative of left or right heart failure, respectively) are normal (see Table 3). As cardiac directed transgenesis could result in activation of the fetal gene program, ANF, α–skeletal actin, and βMHC transcripts of both transgenic and non-transgenic animals were quantified and compared using dot-blot RNA analysis (performed by Amy Canning, laboratory of Dr. Gerald Dorn II, University of Cincinnati 1998). No evidence of upregulation of these genes was observed (data not shown).

Table 3.

Heart and Body weight data for Non-Transgenic (NTG) and Adenylyl Cyclase Type V (ACV) Transgenic Animals. N=15 and 13, respectively. Weights are given in grams (g) or milligrams (mg).

	Body (g)	Heart (mg)	Liver (g)	Lung (mg)	Heart/ Body (g/g)	Liver/ Body (g/g)	Lung/ Body (mg/g)
NTG	25.8± 0.6	144± 6	1.33± 0.06	165± 7	5.6± 0.2	0.05± 0.002	6.4± 0.2
ACV	21.9± 0.8	124± 7	1.06± 0.05	148± 6	5.1± 0.5	0.05± 0.002	6.8± 0.2

After confirmation of the presence of the transgene, animals were screened using three separate methods to determine expression and function of ACV. Western analysis was used to initially identify protein expression (Figure 19a), but, as endogenous AC is difficult to detect, other methods were utilized to confirm and quantitate ACV expression. To more accurately determine the level of overexpression in ACV animals, ^{3}H-forskolin binding was used to quantitatively define total AC number (Figure 19b). This method

detected a 54.4% increase in total AC protein in animals bearing the ACV transgene in both whole heart homogenates (176±18 fmol/mg protein in ACV vs. 114±7 fmol/mg protein in NTG animals, n=4 $p<0.05$) and in isolated myocytes (287±10 fmol/mg protein in ACV animals vs. 189±3 fmol/mg protein in NTG animals, n=2). To confirm that the overexpressed AC was functional, activity was measured in response to forskolin in the presence of manganese. Manganese dissociates Gs from the active site of adenylyl cyclase, removing any synergistic effects in the presence of forskolin. Forskolin/Mn^{2+} responsiveness was increased from 387±30 fmol cAMP/min/mg protein in NTG animals to 492±74 fmol cAMP/min/mg protein in ACV-14.3 animals (n=4, $p<0.05$), and to 640±93 fmol cAMP/min/mg protein in ACV-15.1 animals (n=4, $p=0.05$). (See Figure 19c.)

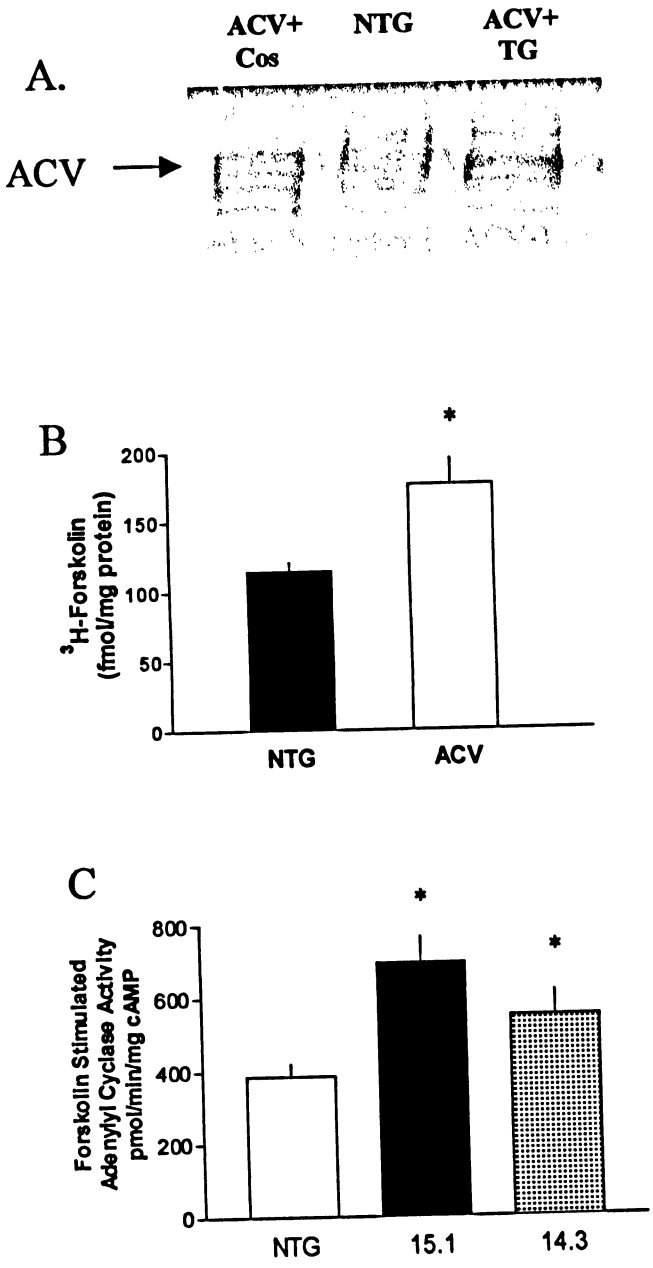


Figure 19. Confirmation of ACV expression using Western Analysis, ^{3}H-Forskolin binding and Forskolin stimulated Adenylyl cyclase Activity. *Panel A: Western analysis in Non-transgenic and ACV Transgenic animals. Panel B: ^{3}H-Forskolin binding in Non-transgenic and ACV Transgenic animals. Panel C: Adenylyl Cyclase activity in response to forskolin (in the presence of manganese) in cardiac membranes of non-transgenic and transgenic ACV animals. *, $p<0.05$ compared to non-transgenic.*

Upon confirmation of ACV overexpression, adenylyl cyclase activity in response to isoproterenol was determined *in vitro*. In the ACV-15.1 animals, basal levels were 45.3±10.8 fmol cAMP/min/mg protein compared to NTG basal activity of 18.6±5.0 fmol cAMP/min/mg protein. ($p<0.05$) Basal levels of activity tended to be higher in ACV-14.3 animals (29.2±5.3), but was not statistically significant. Isoproterenol stimulated AC activity, however, was higher in both ACV lines vs. NTG animals, without a change in EC_{50}. (NTG = 43.8±6.7 vs. 71.0±8.5 in 15.1 animals and 58.6±3.4 in 14.3 animals.) Fold stimulation over basal values was essentially unchanged in ACV-14.3 animals vs. controls, but was decreased in the ACV-15.1 line (Figure 20). All subsequent experiments were performed only with the ACV-14.3.

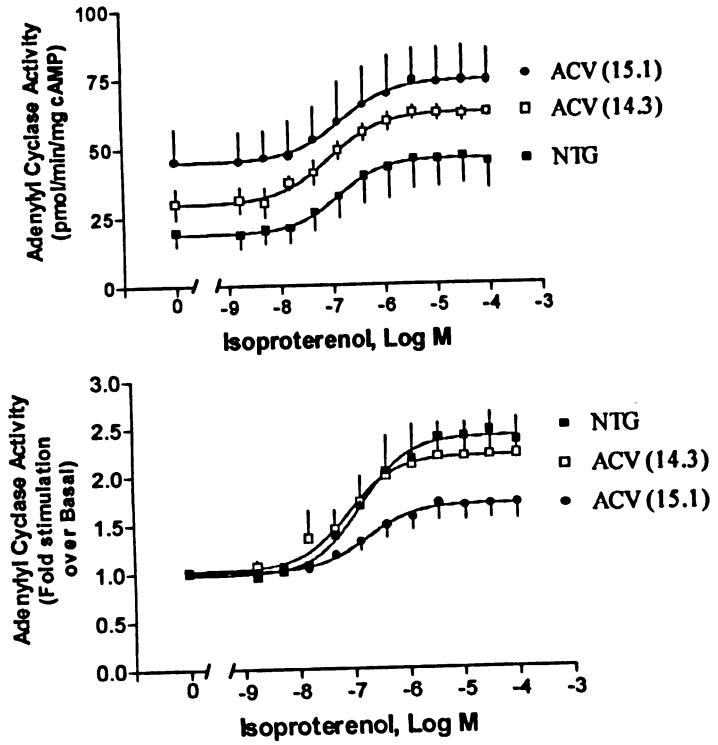


Figure 20. ***Adenylyl cyclase activity in cardiac membranes of non-transgenic and two lines of mice overexpressing adenylyl cyclase type V (ACV).*** *Panel A shows a dose response curve to the beta agonist isoproterenol. Panel B depicts the fold stimulation over basal in response to isoproterenol. Results are means of four separate experiments. See text for statistics.*

With this level of ACV overexpression, basal fractional shortening was enhanced by 20% compared to control animals (n=4-7, $p<0.05$), largely due to a decrease in end systolic dimensions. (See Table 4.) Heart rate was also enhanced in ACV animals to 529±31 bpm from 420±26 bpm of non-transgenic animals ($p<0.05$).

Table 4.

Echocardiograph data for non-transgenic and ACV+ transgenic animals. Data was obtained at 16 weeks of age. Data presented as millimeters ± the standard error of the mean (SE) for EDD and ESD. Heart Rate (HR) presented as beats per minute ± SE. (EDD: End Diastolic Dimension, ESD: End Systolic Dimension, FS: Fractional Shortening, HR: Heart Rate)

	EDD (mm)	ESD (mm)	%FS	HR (bpm)	LVM
NTG	0.37 ±0.02	0.18± 0.01	50 ± 2	420 ± 26	57.3±6.0
ACV+	0.34 ± 0.01	0.13 ± 0.01	61 ± 2	529 ± 31	62.5±5.8

Although enhanced basal cardiac function was observed via echocardiographic measurements, agonist stimulated contractility was not improved versus non-transgenic animals, as determined *in vivo*. Resting heart rate was higher in ACV+ animals (477±21 vs. 412±12 bpm, n=4, $p<0.05$) as previously determined using echocardiography. Maximal heart rate in response to infused dobutamine was no different in ACV animals (579±27 bpm vs. 570±14 bpm in non-transgenic animals.) See Table 5.

Table 5.

In vivo hemodynamics of ACV and non-transgenic animals. Basal values indicate responses without agonist infusion. Maximal values represent 32ng/g/min infusions of dobutamine. (BPM: beats per minute; data is presented as mean ± standard error, n = 4.) * = $p<0.05$ compared to non-transgenic values.

	HR (bpm) Basal	HR (bpm) Maximal	+dP/dt Basal	+dP/dt Maximal
Non-transgenic	412 ± 12	570 ± 14	9300 ± 794	25145 ± 949
ACV+	477 ± 21 *	579 ± 27	10335 ± 748	21441 ± 844

As adenylyl cyclase activity was enhanced without agonist stimulation, basal PKA activity could potentially be increased in cytosolic preparations of ACV hearts contributing to enhanced basal contractility. To investigate this possibility, PKA activity was determined in cytosolic fractions of untreated hearts and hearts treated with 10 uM isoproterenol for five minutes. In non-transgenic hearts, pre-incubation with isoproterenol resulted in nearly three-fold increase in PKA activity compared to untreated hearts. However, in the ACV hearts, PKA activity was maximally activated without agonist treatment, and was equal to that of isoproterenol stimulated levels in non-transgenic mice (See Figure 21A). Because PKA activity was not increased with isoproterenol, though the hearts were clearly responsive to agonist *in vivo* and *in vitro* as measured by adenylyl cyclase activity, PKA activity was assessed in isolated myocytes. In isolated myocytes, basal and maximally stimulated PKA activity was enhanced in ACV animals compared to non-transgenic controls, and isoproterenol mediated increases in activity were observed (Figure 21B) in transgenic myocytes. Fold stimulation over basal with isoproterenol was not as robust as in whole heart homogenates, and may be attributable to a myocyte preparation containing non-viable cells. Maximally activated PKA activity (stimulated by cAMP added to the assay) was not changed between transgenic and non-transgenic animals using either method.

A primary target of PKA in the myocyte that directly alters contractility is phospholamban (PLB). As basal PKA levels in transgenic

ACV animals were increased, it would be expected that unstimulated levels of PLB phosphorylation would also be enhanced. To determine this, Western analysis using a phospho-specific antibody to serine-16 of PLB was used. By this method, basal PLB phosphoryation (in arbitrary units) was increased in ACV animals, from 0.31±0.04 in non-transgenic myocytes to 0.53±0.06 in ACV myocytes ($p<0.05$). Maximally stimulated PLB phosphorylation using isoproterenol was not different between the two groups. This work was done by Raj Dash of the laboratory of Dr. Evangelia Kranias.

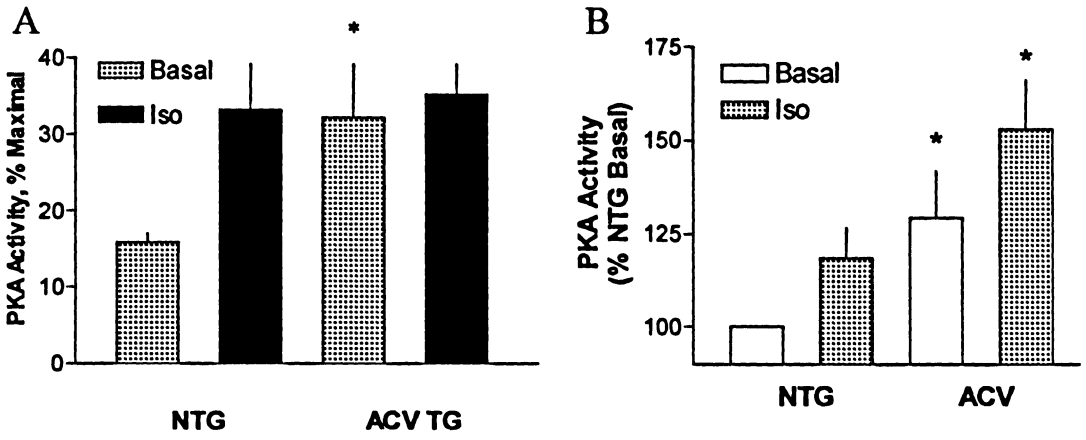


Figure 21. PKA Activity in Cytosolic Preparations of Non-transgenic and ACV whole heart homogenates (A) and isolated myocytes (B). *Increased basal PKA activity was observed in transgenic mice compared to control animals, though PKA activity in ACV animals was not responsive to agonist in whole heart preparations. Studies of PKA activity in myocytes detected an increase in basal and isoproterenol stimulated PKA activity. n=4, * = $p<0.05$ vs. non-transgenic response.*

An additional measure of βAR signalling relevant to the heart is βAR coupling to L-type Ca^{2+} channels. This was determined by patch clamp studies in isolated myoctyes by Dr. Atsuko Yatani, University of Cincinnati.

Consistent with the observation that ACV mice showed no change in heart to body weight or hypertrophy, myocyte size evaluated by cell capacitance was normal in ACV mice. The mean cell capacitance was 128.4±4.0 pF (n=55) and 124.7± 4.0 (n=55) for ACV and NTG cells, respectively. L-type Ca^{2+} current (I_{Ca}) density in AC myocytes were also similar between the two groups: (AC, 13.7±0.9 pA/pF, n=35 vs. NTG, 13.3±0.6 pA/pF, n=51). I_{Ca} in myocytes in response to isoproterenol, was measured in NTG and ACV myocytes. Maximum increases in I_{Ca} amplitude was 216±1% (n=17) of basal levels, and 227±13 (n=18) for non-transgenic and ACV cells, respectively. Thus, from these patch clamp studies we conclude that βAR mediated L-type Ca^{2+} channel activation was not affected by ACV overexpression.

Chapter 7

Results: Adenylyl Cyclase Type V Overexpression in Gαq Animals

The limiting nature of adenylyl cyclase under non-pathological circumstances is not well described. However, in the context of contractile dysfunction, the amount of functional AC at the membrane of the myocyte may be a critical determinant of whole organ physiology and responsiveness to catecholamines. In some human studies of cardiac dysfunction, for example, forskolin responsiveness is blunted. These data suggest that adenylyl cyclase protein is either downregulated or subject to post-translational modification that leads to impaired function. In this context, overexpression of adenylyl cyclase may allow recovery from the desensitized βAR pathway and restore some basal or agonist mediated contractility.

One of the major defects observed in the β-adrenergic signalling pathway with overexpression of Gq, was significantly decreased adenylyl cyclase responsiveness to forskolin. This observed decrease in activity was paralleled by a decrease in ^{3}H-forskolin binding by approximately 50%. Although ^{3}H-forskolin binding cannot differentiate among the various isoforms present in murine cardiac membranes, initial studies of the Gαq animal by Western analysis suggested that the decrease in activity was a result of isoform specific downregulation of AC type V.

To test the hypothesis that the observed downregulation of ACV

contributed to the impaired signalling response *in vivo* and *in vitro*, ACV overexpressing mice were mated with Gαq overexpressing mice. *In vivo* hemodynamics and biochemical parameters were examined in mice carrying both the Gαq and ACV transgenes and compared to both Gαq and non-transgenic animals to better understand the role of adenylyl cyclase in Gq mediated dysfunction.

After identification of animals that expressed both transgenes (ACV and Gαq) using Southern blot techniques, Western blots for Gq were performed on non-transgenic, Gq and ACV/Gαq animals to confirm that overexpression of ACV did not alter Gαq overexpression. Counter regulation of Gq was not observed in the ACV/Gαq animals as assessed by Western analysis (see Figure 22).

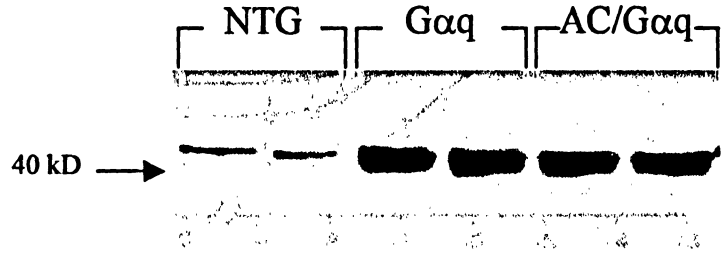


Figure 22. Western Analysis Detecting Gαq Protein in Non-transgenic, Gαq and ACV/Gaq Animals. *Gαq expression was unchanged with transgenic overexpression of ACV.*

Experiments were then carried out to determine the effects of ACV expression on βAR signalling in Gq mice. The most striking feature of the

ACV/Gαq animal was a nearly complete restoration of basal and isoproterenol stimulated adenylyl cyclase activity. Gαq animals, as previously discussed, have a markedly depressed basal and isoproterenol stimulated adenylyl cyclase activity. Although the signalling is only modestly improved with ACV overexpression in non-transgenic animals, the ACV transgene in the Gαq background nearly completely restored basal and isoproterenol stimulated adenylyl cyclase activity to normal levels. (See Figure 23.) Forskolin stimulated adenylyl cyclase activity was also completely restored and, in fact, was slightly higher than that of non-transgenic littermates. However, sodium fluoride stimulated adenylyl cyclase activity was not improved versus Gαq animals. (See Figure 24.)

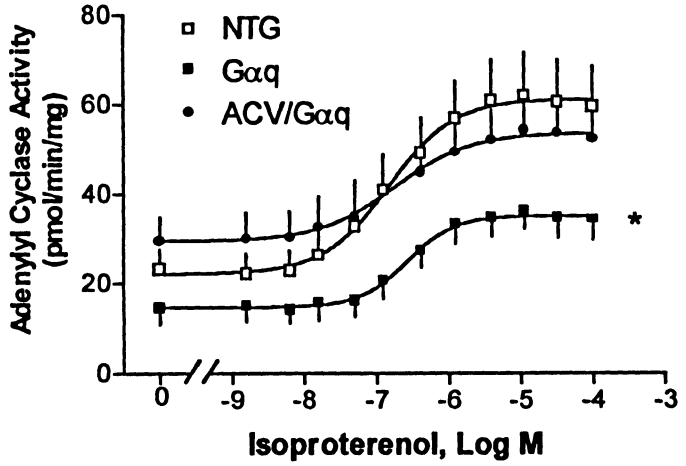


Figure 23. Agonist stimulated adenylyl cyclase activity in non-transgenic, Gαq and ACV/Gαq animals. *Expression of ACV normalized basal and agonist stimulated adenylyl cyclase activity in Gαq membranes.* * = *overall response different than non-transgenic,* $p<0.01$

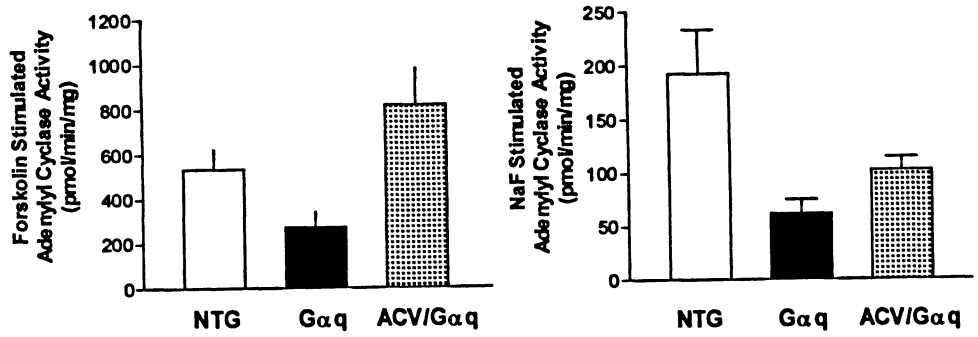


Figure 24. Adenylyl cyclase activity in non-transgenic, Gαq, and ACV/Gαq animals in response to sodium fluoride (panel A) and forskolin (panel B). *Overexpression of adenylyl cyclase in the Gq background restored forskolin stimulated adenylyl cyclase activity, but did not restore NaF stimulated adenylyl cyclase activity. * = overall response different than non-transgenic, $p<0.01$*

Fractional shortening, as measured by echocardiogram, was 46±3% in Gαq/ACV animals (vs. 36±6% in Gαq animals), which was not statistically different from non-transgenic. Heart rate was also increased in ACV/Gαq animals to 388±13 bpm vs. 286±28 bpm (n=5-7, $p<0.05$) in Gαq animals. (Non-transgenic heart rates were 420±26 bpm.) (See Table 6.)

Table 6.

Echocardiograph data for non-transgenic (NTG), Gαq and ACV/Gαq trangenic animals. Data was obtained at 16 weeks of age. Data presented as mm ± the standard error of the mean (SE) for EDD and ESD. HR presented as beats per minute ± SE. (EDD: End Diastolic Dimension, ESD: End Systolic Dimension, FS: Fractional Shortening, HR: Heart Rate). *, $p<0.05$ vs. non-transgenic.

	EDD (mm)	ESD (mm)	%FS	HR (bpm)	LVM
NTG	0.37 ± 0.02	0.18± 0.01	50 ± 2	420 ± 26	57.3±6.0
Gq TG	0.48 ± 0.05 *	0.31 ± 0.06 *	36 ± 6 *	286 ± 28 *	92.0±8.0 *
ACV/Gq	0.36 ± 0.01	0.20 ± 0.02	46 ±3	388 ± 13	74.1±5.6 *

Despite the enhanced heart rate and fractional shortening in ACV/Gαq animals, there was no observable rescue of *in vivo* contractility in response to infused agonist. Neither basal nor dobutamine stimulated +dP/dt was restored in ACV/Gαq mice compared to Gαq mice. Heart rate, in parallel with echocardiographic measurements, was improved in ACV/Gαq animals. ACV/Gαq animals were difficult to sustain under anesthesia. (Figure 25,26).

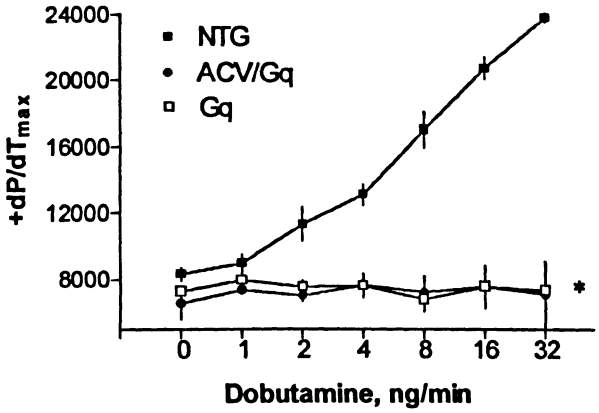


Figure 25. In vivo hemodynamics of non-transgenic, Gαq and ACV/Gαq mice. *+dp/dt in Non-transgenic, Gαq and ACV/Gαq animals in response to infused dobutamine. n=4 for non-transgenics, n=3 for Gαq animals and n=2 for ACV/Gαq animals. Contractility was not improved in Gαq animals with overexpression of ACV. * = overall response different from non-transgenic, $p<0.001$.*

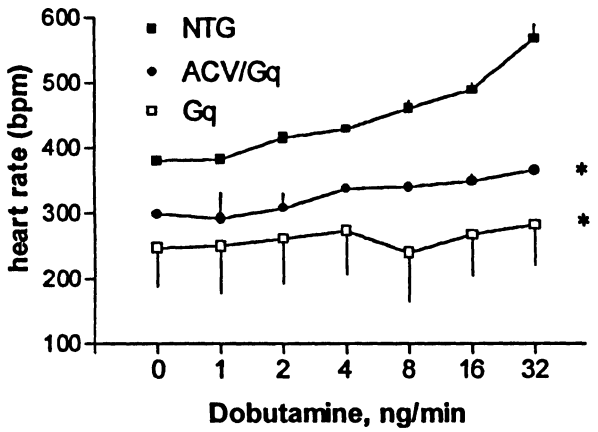


Figure 26. Heart rate in response to infused dobutamine measured in vivo. n=4 for non-transgenics, n=3 for Gαq animals and n=2 for ACV/Gαq animals. Heart rate was improved in Gαq animals with ACV overexpression. * = overall response different from non-transgenic, $p<0.05$.

Preliminary studies of calcium channel kinetics in transgenic and non-transgenic animals revealed a nearly complete restoration of forskolin stimulated calcium channel activity. However, basal and agonist stimulated activation was not improved in ACV/Gq mice compared to Gq mice.

Although improvement βAR signalling through adenylyl cyclase activity was observed, hypertrophy in the Gq mouse was not reversed. Histopathic studies of Gαq mice showed hypertrophic myocytes without inflammation or a consistent degree of significant fibrosis. ACV/Gαq mice showed no improvement in hypertrophy with clear areas of fibrosis (See Figure 27). As shown in table 6, left ventricular mass (LVM) was unchanged in ACV/Gq mice compared to Gq mice, and heart to body weights ratios were also unchanged. (NTG: 8.2±1.0 mg/g; Gq: 11.3±1.0 mg/g; ACV/Gq: 11.1±1.7 mg/g)

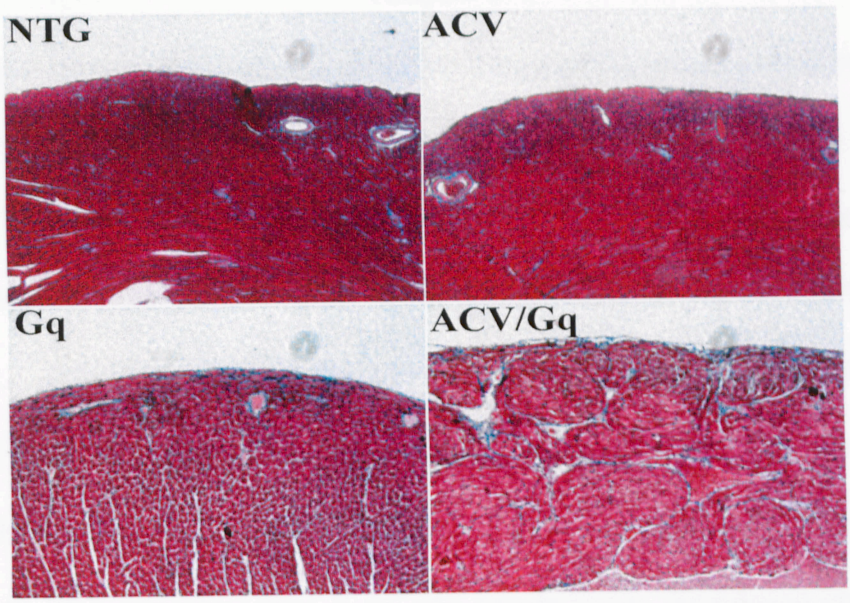
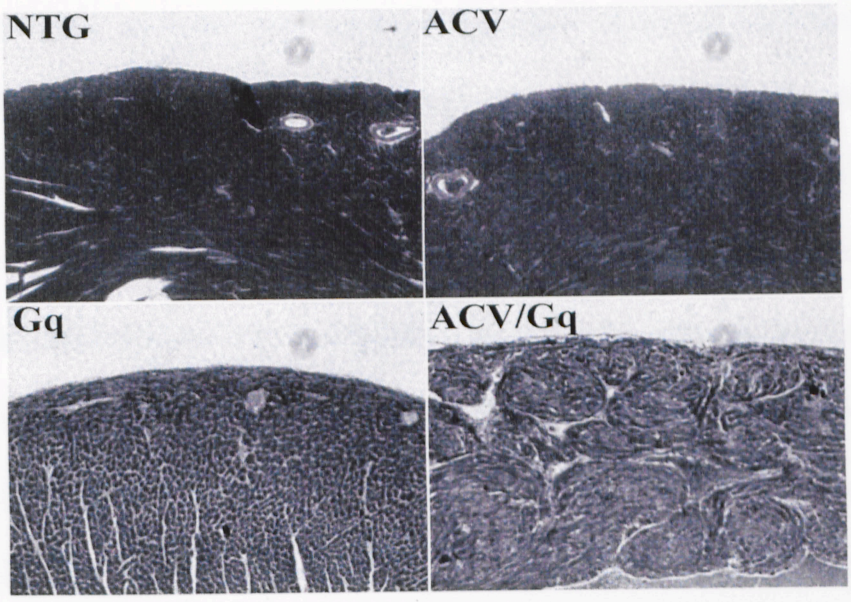


Figure 27: Masson's trichrome stain of myocardial sections from right ventricular free wall of 16 week-old Non-transgenic, ACV, Gq and ACV/Gq animals. *100x magnification. Tissue from Gq/ACV mice display increased fibrosis compared to non-transgenic, ACV and Gq animals.*



Chapter 8

Results: Effects of Gi Overexpression Using a Model Cell Based System

Several studies have suggested that the β_2AR may couple to the inhibitory G protein, Gi. (Xiao et al. 1995; Daaka et al. 1997, Okamoto, 1991). This is contrary to the traditional β-adrenergic receptor paradigm, which holds that this class of receptor couples exclusively to Gs, the stimulatory G protein that activates adenylyl cyclase. Some data suggest that only the PKA phosphorylated β_2AR can couple to Gi. Regulation by Gi may have implications relevant to the failing heart. Upregulation of Gi in the failing heart may result in an imbalance of the ratio of Gi to Gs in the cell, resulting in receptor coupling to Gi or tonic inhibition of adenylyl cyclase activity. This, in turn, may lead to depressed signaling and functional desensitization to agonist. As shown, $G_{\alpha i2}$ and $G_{\alpha i3}$ are increased in the Gαq transgenic mice. However, there are some limitations in studying the effect of β_2AR-Gi coupling, in isolation, in this mouse. Studies investigating the possible consequences of Gi upregulation on β_2AR signalling are described in this work. These were done using cultured HEK293 cells transiently overexpressing the β_2AR alone or the β_2AR and the α subunit of Gi_2. Coupling of β_2AR to the stimulation of adenylyl cyclase was examined, with and without after short-term activation by agonist. If Gi coupling to the β_2AR is significant, a decrease in adenylyl cyclase basal or agonsist simulated activity would be expected. Additionally, the effects of Gi on the high

affinity conformation were investigated using this system. In failing heart animal models, it has been demonstrated that the percentage of receptors in the high affinity receptor conformation (formed through agonist-independent association with Gs) is reduced. To assess a possible role of Gi in altering the high affinity state of the receptor, competition binding was performed on HEK293 cells expressing either both β_2 and Gi, or β_2 alone. This work has the potential to delineate the significance of Gi coupling to the β_2AR and the possible consequences of Gi upregulation in heart failure.

The first method used to discern Gi coupling to the β_2 adrenergic receptor involved the use of pertussis toxin (PTX). HEK-293 cells expressing endogenous β_2AR were treated with toxin and the agonist stimulated adenylyl cyclase activity was determined. Although absolute levels of adenylyl cyclase activity were increased, fold stimulation by isoproterenol over basal was unchanged with PTX treatment. (2.27±0.04 fold in the absence of PTX and 2.40±0.17 fold with PTX treatment, p=NS). However, treatment with pertussis toxin significantly increased absolute levels of adenylyl cyclase activity, making it difficult to compare PTX treated and untreated cyclase responses. Additionally, agonist promoted desensitization could not be determined under these conditions, as the enhanced adenylyl cyclase activity results in increased cAMP levels and PKA activity, contributing to the desensitization of the receptor prior to agonist treatment. To avoid this problem, HEK cells were transiently transfected with β_2AR alone, or with Giα_2. Successful transfection of the receptor was confirmed via radioligand

binding and transfection of Gi was confirmed using Western Analysis (figure 28). If Gi coupling to β_2 occurred, the transfection of Gi would be expected to alter baseline and/or agonist promoted coupling to adenylyl cyclase. As shown in figure 29, expression of Gi did result in decreased agonist responsiveness without pretreatment with agonist. Basal activities were decreased with expression of Gi from 9.06±1.04 to 6.91±1.1 pmol/min/mg, ($p<0.05$, n=4). Maximal isoproterenol responsiveness was also decreased from 23.7±2.2 to 17.9±1.8 pmol/min/mg, ($p<0.05$), but the fold stimulation by isoproterenol over basal was unchanged (2.23±0.12 fold without Gi vs. 2.28±0.16 fold with Gi expression, p=NS). Forskolin stimulated adenylyl cyclase activities tended to decrease with addition of Gi (75.3±6.5 and 60.4±7.5 pmol/min/mg with and without Gi, respectively) but this did not reach statistical significance. (See Figure 30.)

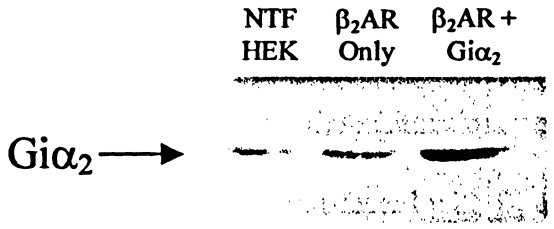


Figure 28. Western Analysis of Gαi expression in HEK 293 Cells. Western Blot analysis determined approximately 5 fold overexpression of Gi.

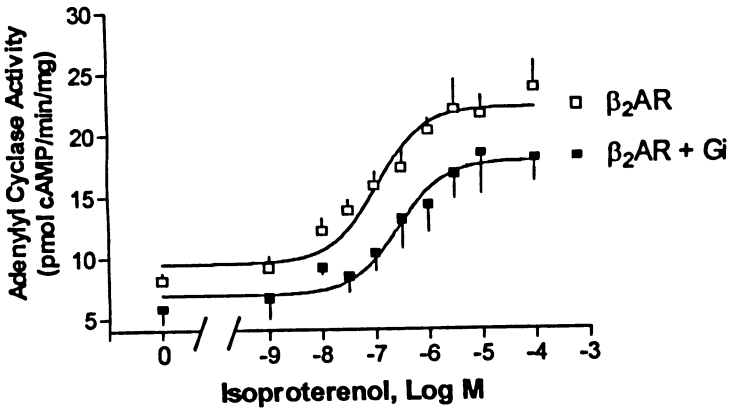


Figure 29. Agonist stimulated adenylyl cyclase activity in HEK 293 cells expressing the α subunit of Gi and/or the β2AR. *Both basal and maximally stimulated adenylyl cyclase activity is decreased with expression of Gi.*

Since β_2AR-Gi coupling has been demonstrated to occur subsequent to PKA mediated phosphorylation of the receptor as a component of the desensitization response, increased Gi might be expected to alter receptor-adenylyl cyclase coupling after pre-exposure to agonist. To determine this, agonist stimulated adenylyl cyclase activity was assessed with and without pre-exposure to agonist. With the increased expression of Gi, agonist pre-treatment resulted in 58±5% desensitization, compared to a 22±7% desensitization response in cells transfected with β_2 alone, ($p<0.01$, $n=4$). (See Figure 31.)

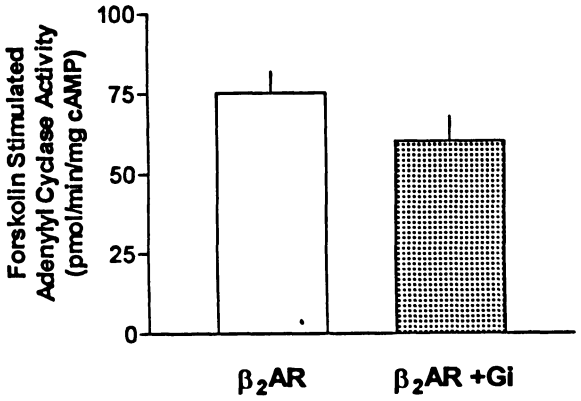


Figure 30. ***Adenylyl cyclase activity in response to forskolin in cells transfected with β_2AR or β_2AR and Giα_2.*** *Maximally stimulated activity (via forskolin) was not different with Gi overexpression compared to cells only expressing β_2AR. P=NS.*

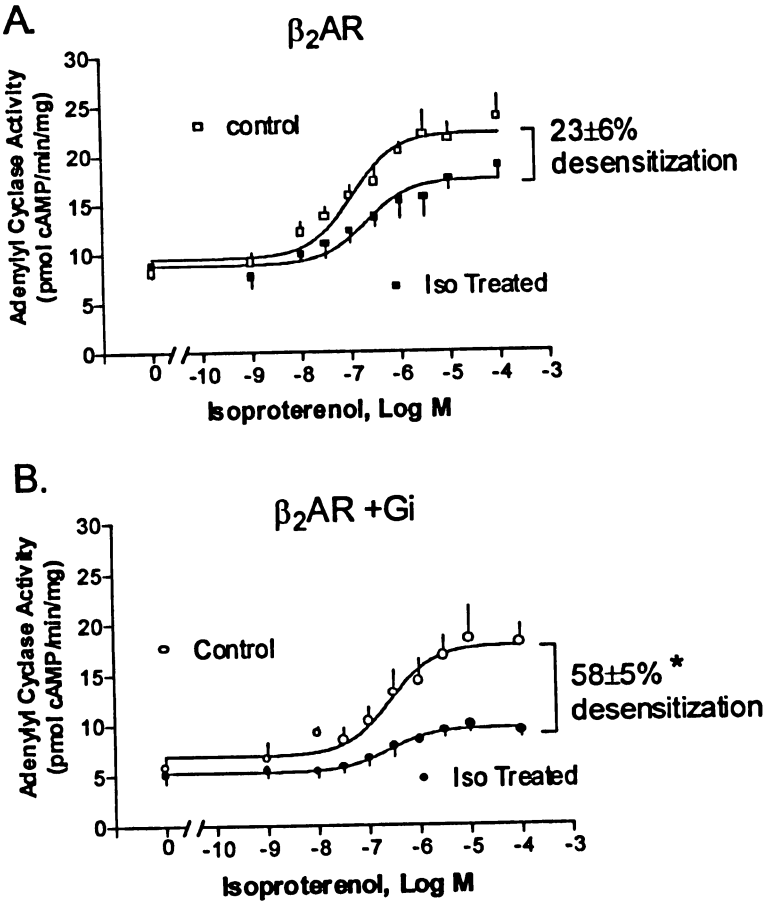


Figure 31. Desensitization Response of β_2AR expressed in HEK Cells with and without Transfection of Giα_2. *Panel A shows adenylyl cyclase activity of HEK cells transiently transfected with β_2AR alone. Panel B shows activity of cells transfected with both β_2AR and Giα_2. *, $p<0.01$ vs. desensitization response of β_2AR transfected cells.*

The effects of upregulated Gi on the formation of the high affinity binding conformation of the receptor were investigated using a similar system to determine if the increased expression of Gi could in fact contribute to the loss of the high affinity conformation observed in the Gαq phenotype.

Competition binding in the absence of GTP was done as described in the methods using HEK-293 cells expressing only β_2AR or β_2AR and Giα_2. Membranes from cells expressing the increased Gi displayed a small but statistically insignificant decrease in the percentage of receptors in the high affinity state ($\%R_H$=14±1.5 vs 18±6.8, n=4, p=NS) without a significant change in binding affinities.

Chapter 9

Discussion

The role of adrenergic modulation of cardiac function in the development of hypertrophy and progression to ventricular dysfunction and heart failure is highly controversial. It is clear that β–adrenergic stimulation is the critical mechanism by which cardiac output is increased as needed in normal physiology and certain pathologic states. As the heart responds to depressed function, though, it is not clear how altered βAR function contributes to the phenotype. Some studies in humans have suggested that attempting to enhance βAR function in patients with heart failure results in untoward effects (Packer, 1990). (The cause of death in these individuals, though, is typically due to arrhythmias rather than progressive failure). Judicious use of βAR antagonists has been shown to increase ejection fraction and prolong survival (Teerlink and Massie 1999, Eichhorn, 1999). Taken together, one concept that has arisen from these studies is that βAR activity is disadvantageous in heart failure.

On the other hand, some animal studies, which utilize methods of increasing βAR signalling that cannot be carried out in humans (such as transgenic expression of a βARK-inhibitor) have shown no deleterious effects on cardiac function in normal mice (Milano et al. 1994, Turki et al. 1996) and improved function in some models of hypertrophy and ventricular dysfunction (Rockman et al. 1998, Dorn et al. 1999). Thus, the manner in which βAR

signalling is altered in a given pathologic state, or enhanced by a given mechanism, may be a critical factor in the characteristics of any cardiac phenotype that may develop.

The mechanistic basis of the depressed function observed in human heart failure and animal models of hypertrophy or ventricular failure is not well understood. Here, we utilized the Gαq overexpressing transgenic mouse, which expressed the α subunit of Gq at five fold non-transgenic levels. Overexpression of the α subunit of Gq in murine hearts results in a phenotype of hypertrophy and depressed basal and β-agonist stimulated contractility (D'Angelo et al. 1997). Animals overexpressing this protein, designated "Gq" mice, were used to investigate the molecular mechanisms that can contribute to impaired β-agonist responsiveness. Studies of human heart disease and animal models of contractile dysfunction have implicated several components of the βAR pathway. Specifically, downregulation of βAR, changes in GRK activity, increases in Gi and changes in adenylyl cyclase have been suggested to contribute to the observed dysfunction.

In these studies, three key alterations in the βAR pathway were identified in the Gq mouse (Dorn et al. 1999). These included an uncoupling of the βAR (likely due to increased PKC), an increase in Gi protein, and a downregulation of adenylyl cyclase protein. These defects are particularly relevant to clinical heart failure, in that these alterations in βAR signalling

have been observed in human studies. Specifically, receptor uncoupling is a well established phenomenon in human failure first observed by Bristow et al. (1982), and increased PKC activity has been characterized in failing human hearts (Bowling et al. 1999) and experimental heart failure (Wang et al. 1995; Mohammadi et al. 1997). Likewise, it has been found that Gi protein in upregulated in failing human hearts 2-3 fold (Feldman et al. 1988) and other animal models of failure (Eshenhagen et al. 1995). Decreased maximal adenylyl cyclase activation (by forskolin) is observed in some animal models (Eschenhagen et al. 1995) and human cardiomyopathies (Reithman et al. 1997).

The primary goals of this work following the identification of these key defects were to address the role of each of these defects in contributing to the severity of blunted agonist responsiveness and hypertrophy. This was accomplished via the generation of dual transgenics designed to compensate for the specific defect, or, in the case of the changes in Gi, using toxin that ablates Gi function *in vivo* and *in vitro*, or model cell based systems in which the stoichiometry of receptor to G protein could be manipulated.

The first of the three defects, the decrease in receptor coupling, was addressed using two separate transgenic models crossed with the Gq mouse. These were: β_2AR overexpressing mice, and mice expressing a βARK-inhibitor peptide (Dorn et al. 1999). Animals heterozygous for the β_2AR were crossed with heterozygous Gq animals to create animals that overexpressed

both the β_2AR and Gq. This was done using several different lines of β_2AR animals, allowing a dose-response effect of the β_2AR on cardiac function of Gq mice. Overexpression of the receptor was attempted to determine the contribution of the observed receptor uncoupling to the overall contractile dysfunction and the impaired adenylyl cyclase activation. In theory, although receptor number in Gq mice was not found to be changed, increased expression of the receptor could replace the pool of receptors that are partially uncoupled from downstream effectors. Receptor levels in the Gq mouse achieved via dual transgenesis were 30-fold, 140-fold and 1000-fold NTG levels. The results of each of the three lines crossed with the Gq mouse were unique. The lowest level of expression (30-fold) resulted in an improved phenotype: increased basal fractional shortening and a decrease in the expression of genes associated with hypertrophy (ANF and α–skeletal actin). No improvement in isoproterenol stimulated contractility was observed. Receptor activation of adenylyl cyclase was not observed *in vitro,* and could account for the lack of agonist stimulated contractility observed *in vivo.* The moderate levels of expression, 140-fold NTG levels, did in fact improve receptor mediated activation of adenylyl cyclase, demonstrating that cyclase protein could be activated when sufficient numbers of βAR was present. Such overexpression, though improving receptor signalling at the membrane, resulted in a phenotype of increased hypertrophy and no improvement in basal contractility. The highest level of expression, 1000-fold NTG levels, resulted in massive cardiomegaly and early death. Thus, in the context of compromised function of Gq mice, chronic increases in adenylyl cyclase

activity achieved with β_2AR overexpression results in myocardial decompensation. These results are similar to those observed by Rockman et al (1998) in that lethal effects were observed when the β_2AR was overexpressed in the MLP knockout mouse.

Although the exact mechanism by which the β_2AR contributes to hypertrophy gene expression or contractility could not be determined with these studies, it is interesting to speculate the reasons for the dose-dependent phenotypes observed in the Gq animals. Also note-worthy is the observed dissociation between receptor mediated adenylyl cyclase activation and function. As discussed, at the lowest level of expression, an improvement in function was observed without an increase in adenylyl cyclase activation. This suggests two possible scenarios: 1) that AC activation does occur, but in a compartmentalized manner that cannot be measured using the reported method (Zhou, et al, 1997), or 2) the β_2AR couples to alternate pathways that have a beneficial effect on hypertrophy and/or contractility. β_2AR have been shown to activate MAP kinase through coupling to Gi (Daaka et al. 1998), and directly couple to the Na^+/H^+ exchanger (Hall et al. 1998), affecting proton exchange. However, MAP kinase was not found to be increased with β_2AR overexpression alone, or with co-expression of Gq. At higher levels of expression achieving receptor mediated adenylyl cyclase activation, these beneficial effects are lost. Although the reasons for this are not clear, it may be possible that higher levels of expression result in promiscuous coupling of the receptor to alternate pathways that negate the benefits observed at lower

expression levels. Likewise, in the highest expressing line, promiscuous coupling or excessive energy demand due to the extremely high expression could be the cause of massive hypertrophy and failure.

A substantial body of literature has accumulated in the past several years questioning the benefit of sustained sympathetic stimulation. However, it is important to consider the differences observed in transgenic studies that activate this pathway. β_2AR overexpression results in a dramatically different phenotype than that observed with sustained Gs activity or overexpression of the β_1AR. Gs overexpression (which shows minimal, if any, increases in adenylyl cyclase activity) results in a phenotype of cardiomyopathy with senescence. β_1AR overexpression, conversely, results in a dramatic phenotype of marked myocyte hypertrophy and progressive failure (Englehardt et al. 1999) at relatively low levels. However, the current studies show favorable effects may be achieved with overexpression of β_2AR at levels that presumably preserve the specificity of signalling.

Expression of a peptide designed to inhibit βARK activation, the "βARK-minigene", was used to attempt to improve function in the Gq mice by inhibiting receptor uncoupling occurring as a result of βARK mediated phosphorylation. These experiments were carried out because of the data in human studies that show that βARK levels are increased, and the results of transgenic studies showing that βARK inhibition increases cardiac

contractility. Subsequently, βARK-minigene expression in the MLP-knockout mouse was shown to improve cardiac function. In the Gαq mouse, however, expression of the βARK-minigene had no effect on cardiac function. This is consistent with the fact that βARK levels or GRK activities are not increased. (Any effect of the minigene on existing GRK activity was obviously not significant.) The effects of this peptide might also have been compromised by Gαq overexpression. The βARK minigene exerts its effect by binding to free βγ subunits. With the excess Gαq subunits present in the Gq mouse, free βγ might be limited, thereby limiting the effectiveness of the minigene. Taken together, these results from our studies and the others cited suggest that cardiac βAR dysfunction in "lesion-specific" and that different models likely have different mechanisms causing impaired βAR signalling.

The defect observed in adenylyl cyclase protein in the Gq mouse was also addressed using dual transgenesis. Additionally, the role of adenylyl cyclase in βAR signalling under normal physiological conditions was explored independently of the Gq mouse. Changes in AC expression and/or function have been documented in human and animal forms of cardiac dysfunction. However, neither the role of AC expression in dysfunction or under normal physiological conditions has been well explored. Recent work by Gao, et al. (1999) has suggested that AC protein limits the βAR signalling pathway. This hypothesis is supported by data defining the stoichiometry of receptor:Gs:AC to be 1:200:3 (Post et al. 1995). To address this possibility,

mice were generated expressing the type V Adenylyl cyclase protein at levels approximately 50% over non-transgenic levels. Type V was chosen as it was the isoform found to be downregulated in Gq animals, and is the predominant isoform of the human myocardium (Raimundo et al. 1999).

Our studies of ACV overexpressing mice demonstrated that β-adrenergic receptor signalling is not, in fact, limited by the levels of AC. Although maximal activated activity was increased with AC overexpression, basal levels were also increased, and the overall signalling response was not enhanced. Likewise, measurements of downstream targets of βAR stimulation were not enhanced versus non-transgenic animals: fold PKA activity over basal was not increased, maximally activated phospholamban phosphorylation was not increased, nor was calcium channel activation. *In vivo* measurements of contractility (basal or agonist stimulated) were not enhanced with ACV overexpression. This work is in contrast to data reported by Gao, et al (1999) in which the Type VI isoform was expressed 30-fold over non-transgenic levels. In this study, overexpression of this isoform resulted in a significant increase in agonist activation of adenylyl cyclase, and a significant increase in agonist mediated contractility and heart rate. This occurred without a change in basal adenylyl cyclase activity or *in vivo* resting cardiac function. The authors concluded, from these data, that adenylyl cyclase expression has no effect on transmembrane signalling, except when the receptors are activated. Our findings, however, suggest that the levels of AC set the baseline for adrenergic signalling, but do not enhance the

signalling response.

The reasons for this contradiction may lie in the difference in the isoform expressed. In our study, type V was used, whereas type VI was used in the Gao study. Although there is approximately 85% amino acid homology between the two isoforms, it is likely that there are marked differences in the regulatory properties of these two proteins. It is known that Type V and VI have sequence variability with respect to the number of putative PKA phosphorylation sites (Iwami et al. 1995), which may contribute to the differences observed *in vivo*. However, PKA regulation cannot account for *in vitro* differences, as adenylyl cyclase assays were performed with washed membranes. Additionally, it is well established that these two isoforms can be differentially regulated by PKC isoforms (Kawabi et al. 1994). This becomes especially critical in the context of the Gq mouse, in which PKC isoform expression is changed. Finally, expression levels were dramatically different in the two studies. Our studies utilized animals expressing 50% over non-transgenic levels, whereas the Gao study used animals expressing 30-fold non-transgenic levels.

With these studies, the role of ACV in non-pathological conditions was addressed. However, ACV expression may become critical to normal functioning within the context of dysfunction. The ACV mouse, was then used to determine the role of decreased AC in the Gq mouse. As reported in this work, adenylyl cyclase protein was found to be decreased by

approximately 50% as determined by ^{3}H-forskolin binding and maximal activity. Western Analysis suggested that this was an isoform-specific event, as only the type V isoform appeared to be decreased in Gq cardiac homogenates. To compensate for the 50% decrease in AC protein observed, transgenic ACV mice (expressing 50-75% of non-transgenic levels) were mated to Gq animals. Generation of dual transgenics (ACV/Gq mice) was achieved, and assessment of function allowed an estimate of the contribution of decreased AC protein to the Gq phenotype.

ACV expression, confirmed by forskolin stimulated activity, resulted in a restoration of basal and isoproterenol stimulated adenylyl cyclase activity. Additionally, basal *in vivo* fractional shortening and heart rate (as assessed by echocardiography) was improved compared to Gq mice. However, *in vivo* hemodynamics determined by invasive cardiac catheterization were not improved in ACV/Gq mice compared to Gq mice, and there was no responsiveness to infused catecholamines. Likewise, hypertrophy was also not improved in Gq mice with ACV expression. So, restoration of this protein had a beneficial effect on receptor signalling to AC and basal contractility, but did not improve agonist stimulated function or hypertrophy.

In the ACV/Gq, in contrast to the β_2AR/Gq dual transgenics, improvement of adenylyl cyclase activation was easily achieved, but was not accompanied by a reversal of hypertrophy. This suggests, again, that the β_2AR has unique signalling properties that influence hypertrophy independent

of AC activation, and that AC activation is dissociated from the hypertrophy response. This is unlike the published studies of Roth, et al (1999), in which ACVI overexpression in the Gq background resulted in enhanced basal and agonist stimulated function accompanied by a decrease in some hypertrophy gene expression. Again, this is likely due to the differences in potential regulatory properties of these two isoforms, which is especially critical in the context of the Gq mouse where PKC isoforms are differentially regulated.

The final lesion addressed in the Gq mouse was the upregulation of Gi protein. This defect is relevant to studies of human heart failure, in which Gi protein is upregulated 3-4 fold that of non-failing hearts (Feldman et al. 1988). This was done using pertussis toxin (PTX) to ablate Gi function in vivo and in isolated myocytes. Interestingly, animals treated with PTX *in vivo* did not survive, whereas the dose was well-tolerated in non-transgenic animals, suggesting that the increase in Gi, or perhaps the function of Gi coupled receptors is a compensatory mechanism in the Gq animals. To circumvent this problem and address the consequences of Gi on signalling in the Gq mouse, isolated myocytes were utilized. Using this method, Gi ablation via PTX resulted in enhanced basal and isoproterenol stimulated AC activation in both non-transgenic and Gq mice. However, this increase in activity in the Gq mice did not approach that of non-transgenic values. Fold stimulation over basal, however, was increased in Gq myocytes compared to control myocytes. This work suggests that the increase in Gi does have an effect on signalling in the myocyte, but the effects are minimal.

Although the effects of Gi on the overall pathology of the Gq mouse was determined to be minimal, it is interesting to speculate the effects of Gi on βAR signalling and AC activation. As mentioned, coupling of the β_2AR to the inhibitory G protein Gi has been demonstrated in several studies (Daaka et al. 1997; Okomoto et al. 1991; Xiao et al. 1995,1999). In the study by Daaka and in that by Okomoto, this coupling occurred subsequent to PKA mediated phosphorylation of the β_2AR. What has not been determined with these studies, however, is the effects of receptor-Gi coupling on the activation of adenylyl cyclase. To address this, a model cell based system was utilized in which the stoichiometry of receptor to Gi protein could be manipulated. As presented here, Gi overexpression had a tonic inhibitory effect on signalling under basal conditions, decreasing both basal and isoproterenol stimulated AC activation. This occurred without a change in fold stimulation over basal, suggesting that the coupling of the βAR was not changed with Gi expression. However, under conditions of desensitization, where PKA is activated, increased Gi expression attenuates the activation of AC through the βAR. In fact, Gi expression of the same magnitude as that observed in the Gq mouse results in a desensitization response nearly three times that observed in membranes with normal levels of Gi protein. Upregulated Gi, such as that observed in human heart failure, could regulate βAR signalling under conditions where the receptor is subject to desensitization (e.g. via circulating catecholamines). Its relative contribution to βAR dysfunction in the Gαq mouse, though, appears to be small. And, given that isolated human myocyte studies have not been carried out, it is not clear whether the increased Gi that

is observed in failure is an epiphenomenon or has a causative role in βAR dysfunction.

In conclusion, these studies have explored the role of three defects in βAR signalling that have been observed in both human and animal models of cardiac dysfunction. As these data clearly show, the alterations in signalling that result from Gαq activation are complex, and changes in different proteins have varying influences on the phenotype. The complexity of these interactions suggest that a better understanding of these pathways and the mechanisms involved will lead to more suitable pharmacological or genetic therapies that will better target specific points of dysfunction, and lead to more successful therapeutic approaches to cardiac hypertrophy and failure.

Chapter 10

Conclusion

In summary, cardiac specific overexpression of the α subunit of Gq results in an overt phenotype of cardiac hypertrophy and contractile impairment that is marked by a virtually absent response to β-agonists. The current studies were carried out to determine the primary defects that contribute to this dysfunction. These studies were conducted in a manner designed to identify the major proteins of the βAR pathway which were altered, then assess the relative contribution of the defects to the overall phenotype using transgenesis and cell-based studies.

In this work, the major defects observed to contribute to the depressed β-agonist responsiveness as a result of Gαq overexpression were an increase in Gi protein, an uncoupling of the β-adrenergic receptors, (likely via PKC mediated phosphorylation), and a decrease in adenylyl cyclase protein expression/function.

As this work demonstrates, the changes that occur appear to affect the pathology of the Gq mouse in specific ways. This was determined via transgenic manipulation of the Gαq mouse. Overexpression of the β_2AR, designed to overcome receptor uncoupling in the Gαq mouse, resulted in a partial restoration of contractility and a reversal of hypertrophy without improvement of βAR mediated adenylyl cyclase activation, suggesting non-

cAMP dependent mechanisms that contribute to improved physiology. Moderate levels of receptor expression, however, that achieved significantly enhanced agonist stimulated adenylyl cyclase activity, resulted in increased expression of hypertrophy associated genes and a reversal of functional improvements observed at lower levels of receptor expression.

As decreased adenylyl cyclase protein/function was observed in the Gaq animal, studies were conducted to address the role of adenylyl cyclase protein in normal mice and the Gαq mice. The stoichiometry of receptor to G protein to adenylyl cyclase (AC) has been reported to be 1:200:3, suggesting that AC protein may limit the signal transduction cascade through the βAR in the normal heart. To test this hypothesis, the predominant AC isoform in the heart (Type V) was overexpressed in murine cardiomyocytes, and basal and βAR agonist stimulated function was assessed *in vivo* and *in vitro*. Overexpression was well tolerated in non-transgenic animals and did not result in any untoward effects. Despite increased levels of AC protein, however, βAR signalling was not enhanced.

Replacement of adenylyl cyclase levels in the Gq animal, conversely, allowed a complete restoration of *in vitro* βAR activation of adenylyl cyclase, suggesting that a 50% *decrease* in AC protein could in fact limit βAR signalling. Basal fractional shortening in Gαq animals overexpressing AC was not different from non-transgenic controls. Although signalling through AC was improved, AC overexpression did not reverse Gαq mediated

hypertrophy or enhance agonist stimulated contractility *in vivo*.

The third defect investigated was the upregulation of Gi. As Gαq animals could not be sustained after complete ablation of Gi using pertussis toxin (though the same dose was well tolerated in non-transgenic animals), isolated myocytes and a cell-based model system were utilized. As a result of this work, it was found that the increases in Gi had a small effect on βAR coupling to adenylyl cyclase. However, under conditions of persistent agonist exposure, β_2AR-Gi coupling became functionally significant and increases in Gi protein clearly altered receptor signalling to adenylyl cyclase. Thus, an increase in Gi in the heart plays a role in the Gαq phenotype, although it is not the predominant mechanism.

These findings suggest several key interfaces in the β-adrenergic signal transduction cascade that contribute to the phenotype and may be amenable to pharmacologic or genetic therapy.

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