Hybrid Transgenic Mice Reveal In Vivo Specificity of G Protein–Coupled Receptor Kinases in the Heart

Andrea D. Eckhart, Sandra J. Duncan, Raymond B. Penn, Jeffrey L. Benovic, Robert J. Lefkowitz, Walter J. Koch

Abstract—G protein–coupled receptor kinases (GRKs) phosphorylate activated G protein-coupled receptors, including α_{1B}-adrenergic receptors (ARs), resulting in desensitization. In vivo analysis of GRK substrate selectivity has been limited. Therefore, we generated hybrid transgenic mice with myocardium-targeted overexpression of 1 of 3 GRKs expressed in the heart (GRK2 [commonly known as the β-AR kinase 1], GRK3, or GRK5) with concomitant cardiac expression of a constitutively activated mutant (CAM) or wild-type α_{1B}AR. Transgenic mice with cardiac CAMα_{1B}AR overexpression had enhanced myocardial α_1AR signaling and elevated heart-to-body weight ratios with ventricular atrial natriuretic factor expression denoting myocardial hypertrophy. Transgenic mouse hearts overexpressing only GRK2, GRK3, or GRK5 had no hypertrophy. In hybrid transgenic mice, enhanced in vivo signaling through CAMα_{1B}ARs, as measured by myocardial diacylglycerol content, was attenuated by concomitant overexpression of GRK3 but not GRK2 or GRK5. CAMα_{1B}AR-induced hypertrophy and ventricular atrial natriuretic factor expression were significantly attenuated with either concurrent GRK3 or GRK5 overexpression. Similar GRK selectivity was seen in hybrid transgenic mice with wild-type α_{1B}AR overexpression concurrently with a GRK. GRK2 overexpression was without effect on any in vivo CAM or wild-type α_{1B}AR cardiac phenotype, which is in contrast to previously reported in vitro findings. Furthermore, endogenous myocardial α_1AR mitogen-activated protein kinase signaling in single-GRK transgenic mice also exhibited selectivity, as GRK3 and GRK5 desensitized in vivo α_1AR mitogen–activated protein kinase responses that were unaffected by GRK2 overexpression. Thus, these results demonstrate that GRKs differentially interact with α_{1B}ARs in vivo such that GRK3 desensitizes all α_{1B}AR signaling, whereas GRK5 has partial effects and, most interestingly, GRK2 has no effect on in vivo α_{1B}AR signaling in the heart. **(*Circ Res*. 2000;86:43-50.)**

Key Words: desensitization ■ adrenergic receptors, α_1 ■ protein-coupled receptor kinase ■ myocardial biology ■ myocardial hypertrophy

The α_{1B}-adrenergic receptor (AR) is a member of the G protein–coupled receptor family and is the predominant α_1AR subtype expressed in adult rodent myocardium.[1,2] α_1AR agonists, including phenylephrine (PE), have been shown to mediate intracellular responses through α_1AR activation of the heterotrimeric G protein Gq, which in turn activates the effector enzyme phospholipase C (PLC). Activation of the α_1AR-Gq-PLC pathway results in the cellular accumulation of inositol 1,4,5-trisphosphate and diacylglycerol (DAG), leading to increased intracellular calcium and protein kinase C activity.[3] The role of α_1ARs in the heart is not well understood; however, the Gq-PLC–protein kinase C pathway is important in initiating the hypertrophic response.[4] In fact, we have recently shown, using transgenic (Tg) mice, that signaling through Gq is the final common trigger of in vivo pressure overload ventricular hypertrophy.[5]

Activation of α_1ARs in cultured neonatal ventricular myocytes has been shown to induce an embryonic program of gene expression, including ventricular expression of atrial natriuretic factor (ANF), and cell hypertrophy without hyperplasia.[6,7] Moreover, adult Tg mice expressing a constitutively activated mutant (CAM) of the α_{1B}AR in a cardiac-specific manner have elevated myocardial DAG content, ventricular ANF expression, and myocardial hypertrophy, as measured by increased heart-to-body weight ratio and myocyte cross-sectional area.[8] Thus, constant stimulation of the α_{1B}AR in vivo is sufficient to induce a hypertrophic phenotype independent of hemodynamic changes.

Signaling through α_1ARs is regulated, like that mediated by many other G protein–coupled receptors, via phosphorylation and the triggering of desensitization mechanisms.[9] Phosphorylation of agonist-occupied α_1ARs is accomplished by members of the serine/threonine G protein–coupled receptor kinase (GRK) family. Three predominant GRKs are expressed in the mammalian heart, as follows: GRK2 (com-

Received October 6, 1999; accepted October 20, 1999.

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Transgenic Mouse Lines Used in the Present Study

Parental Lines, Mouse	Description	Level of Myocardial Overexpression (Fold Overexpression Versus Endogenous Levels)	References
1. CAMα_{1B}AR	Constitutively activated mutant of the α_{1B}AR	3	8
2. WTα_{1B}AR	Wild-type α_{1B}AR	40	18
3. GRK2-3	Bovine GRK2 (βARK1)	≈3	15, 16
4. GRK2-20	GRK2	≈20	
5. GRK3	Bovine GRK3 (βARK2)	≈12	17
6. GRK5	Bovine GRK5	>30	16
7. GqI	Minigene of last 54 amino acids of murine Gαq(305–359)	NA	5
Resultant Hybrid Lines			
1. CAMα_{1B}/GRK2-3	3. CAMα_{1B}/GRK3	5. CAMα_{1B}/GRK/GqI	7. WTα_{1B}/GRK5
2. CAMα_{1B}/GRK2-20	4. CAMα_{1B}/GRK5	6. WTα_{1B}/GRK2-3	8. WTα_{1B}/GqI

monly known as the β-AR kinase [βARK1]), GRK3 (βARK2), and GRK5.[10] Although many studies have been done in vitro concerning the actions of GRKs on receptor signaling, almost nothing in vivo has been done to elucidate substrate specificity of GRKs. In fact, in vitro studies with several receptor systems important in the heart have revealed no definitive GRK selectivity.[11–14] In vitro studies examining α_{1B}ARs have found that GRK2 and GRK3 could both increase agonist-induced phosphorylation of the α_{1B}AR and promote desensitization of signaling, whereas GRK5 increased the basal phosphorylation of α_{1B}ARs without any effect of agonist-stimulated phosphorylation.[12] More recently, however, GRK substrate selectivity has been identified in vitro, as Iacovelli et al[15] described the way in which GRK2 stably transfected into rat thyroid FRTL-5 cells desensitized endogenous thyrotropin receptors, whereas GRK5 and GRK6 did not. Additionally, GRK2 had variable effects on the Gαi-coupled A_1 adenosine receptor. Adenylate cyclase inhibition was unaffected, but mitogen-activated protein kinase (MAPK) signaling was attenuated by GRK2 overexpression.[15] Importantly, and in contrast to Diviani et al,[12] Iacovelli et al[15] also found that GRK2 overexpression did not alter α_{1B}AR signaling. Thus, this suggests that there may be cell-type specificity of the GRKs for their various substrates, making it essential to understand the in vivo selectivity of these kinases in the appropriate cells/tissues of interest.

Recently, the use of Tg mice with cardiac-specific overexpression of GRK2, GRK3, or GRK5 have made it possible to address in vivo, tissue-specific GRK substrate selectivity.[16–18] The CAMα_{1B}AR and GRK Tg mice provide a unique and powerful opportunity to create hybrid Tg mice to study the specific interactions in vivo between these 3 individual GRKs and α_{1B}ARs. In the present study, the hearts of these hybrid mice were used as novel "in vivo reaction vessels" to determine the in vivo selectivity of GRK2, GRK3, and GRK5 for myocardial α_{1B}ARs. Furthermore, these mice were used to elucidate a possible role of α_{1B}AR desensitization in the control of myocardial hypertrophy. These results reinforce that it is essential to verify in vitro findings in vivo in the context of the whole organism to begin to understand the true complexity of the mammalian cardiovascular system.

Materials and Methods

Experimental Animals

Myocardial-specific overexpression of the corresponding transgene was targeted by the α–myosin heavy chain (αMyHC) gene promoter as previously described.[19] Each of the single-Tg mouse lines used as parental crossbreeders has previously been described as noted in the Table, except for GRK2-20. All Tg lines were originally generated in C57BL/6 mice. To generate the GRK2-20 mouse, a GRK2 Tg construct was prepared by inserting the complete open reading frame of bovine GRK2 into pGEM containing a 5.5-kb *Sac*I/*Sal*I fragment from the αMyHC promoter and the simian virus 40 intron–poly A. Tg animals were screened by Southern blot and polymerase chain reaction analysis of tail clip DNA.

Hybrid double-Tg mice were created by mating single-Tg mice together as described in the Table. The animals in this study were handled according to approved protocols and animal welfare regulations at Duke University Medical Center.

Radioligand Binding

Tg mouse heart crude membranes were prepared as described.[19] For determination of myocardial αAR binding density, 250 pmol/L (±)-β-([^{125}I]Iodo-4-hydroxyphenyl)-ethyl-aminomethyl-tetralone (^{125}I-HEAT; New England Nuclear) in the absence (total binding) or presence (nonspecific binding) of 50 μmol/L prazosin was used.[20]

Protein Immunoblotting

Immunodetection of GRK2 and GRK3 was carried out first with an immunoprecipitation using a monoclonal GRK2/GRK3 antibody, as described previously.[18] GRK5 was detected in crude membrane protein extracts.[17]

Heart Weight–to–Body Weight Ratio

Mice were first anesthetized[21] and weighed, and their hearts were quickly excised, blotted dry, weighed, and frozen in liquid N_2. Heart weight–to–body weight ratios were calculated and expressed in mg/g.[21]

Ventricular ANF mRNA Analysis

The apical portion of the left and right ventricles of frozen hearts obtained as described above was homogenized, total RNA was extracted using an Ultraspec solution (Biotecx Laboratories), and Northern analysis was performed as previously described.[8,20] After ANF detection, all blots were stripped and reprobed with a rat

GAPDH cDNA probe. The ANF and GAPDH bands were quantified with a PhosphorImager (Molecular Dynamics), and the ANF/GAPDH signal intensity was determined.[20]

DAG Quantification

Lipid fractions from frozen hearts were extracted as described.[8,20] ^{32}P-labeled phosphatidic acid (phosphorylated DAG) was isolated by silica gel thin-layer chromatography and quantified with the PhosphorImager. DAG content was normalized to tissue phospholipid, and the final DAG concentration was expressed as pmol of DAG/nmol of lipid phosphate, as described previously.[8,20]

MAPK Activity

Mice were given a 200-μL intraperitoneal injection of 500 μmol/L PE or saline. After 10 minutes, mice were anesthetized as described above and hearts were quickly removed and frozen. Excised hearts were prepared as described previously.[5,18] Immunoprecipitations were performed using anti–extracellular signal–regulated kinase (ERK2) or anti–c-*jun* N-terminal kinase (JNK1) antibody (Santa Cruz Biotechnology). Kinase assays were carried out at 30°C for 15 minutes[5,18] using myelin basic protein (MBP) (for ERK2) with glutathione *S*-transferase–c-*jun* (JNK1) as a substrate.

Statistical Analysis

Data are expressed as mean±SEM. An unpaired 2-tailed Student *t* test was performed for all biochemical data. For all analyses, $P<0.05$ was considered statistically significant.

Results

Transgene Expression

The Table lists the 15 lines of myocardium-targeted Tg mice used throughout this study, including parental single-Tg mice and the resultant hybrid double-Tg offspring. Included is the documented overexpression levels compared with the endogenous myocardial proteins. Two separate lines of GRK2 animals were used; one line, GRK2-3, has been previously described,[16,17] and the other is an as-yet-unreported GRK2 mouse line, GRK2-20, with ≈20-fold enhancement of GRK activity as compared with endogenous GRK2 activity. Figure 1A illustrates the relative kinase activity that each of the single-Tg lines has in comparison with the others versus non-Tg littermate control (NLC). The primary GRK activity in the mouse heart is GRK2[22]; however, all of the kinases are equally capable of phosphorylating rhodopsin. Each transgene constitutes the majority of activity when overexpressed in the heart. Whereas the Table describes overexpression levels of the various GRKs with respect to their endogenous levels, when expressed in comparison with total NLC GRK activity, Tg GRK2-3 has ≈3-fold, TgGRK2-20 has ≈20-fold, TgGRK3 has ≈5-fold, and TgGRK5 has ≈20-fold overexpression in activity (Figure 1A). Hybrid Tg mice were examined to verify that both transgenes were expressed at equivalent levels as found in the single-Tg parental lines. Importantly, we found no change in expression in any of the transgene protein products in hybrid mice. For example, as shown in Figure 1, protein immunoblotting revealed no difference in the level of overexpression of GRK2 or GRK3 when comparing hearts from GRK2-3 or GRK3 with CAMα_{1B}/GRK2-3 and CAMα_{1B}/GRK3 hearts (Figure 1B). Similar results were found in hybrid Tg mice overexpressing GRK5 (Figure 1C) and the GqI (data not shown). Furthermore, endogenous GRK levels were not altered in the

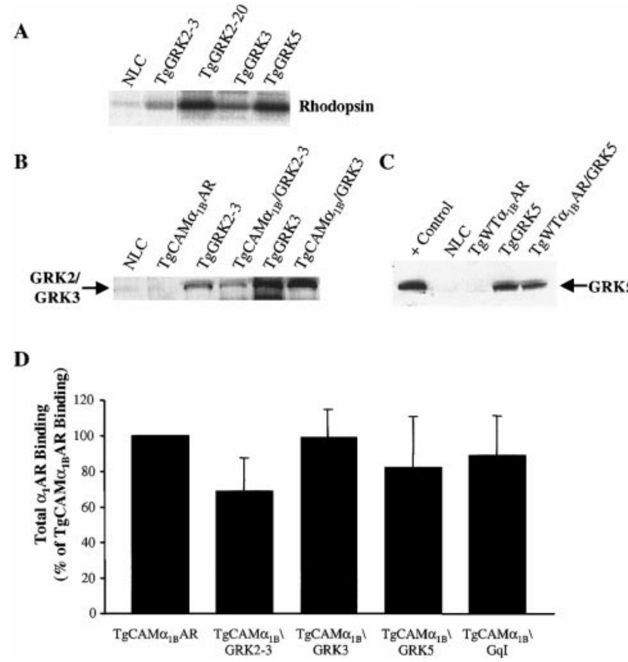

Figure 1. Hybrid transgene expression did not alter single-transgene expression. Cytosolic and membrane proteins were purified as described in Materials and Methods. A, Rhodopsin phosphorylation assays to determine relative kinase activity between the various GRK-overexpressing Tg lines. Primary GRK activity in the NLC is GRK2.[22] B, Immunodetection of myocardial levels of GRK2-3 or GRK3 from cytosolic NLC mice and from single-Tg and hybrid Tg mice as indicated. C, Immunodetection of membrane-associated GRK5 in NLC and in single-Tg and hybrid Tg mice using a selective GRK5 antibody and a recombinant positive (+) control. D, Myocardial α_1AR density was determined using membranes from CAM$_{\alpha1B}$AR and hybrid Tg mice and expressed as percentage of CAM$_{\alpha1B}$AR overexpression. Data are expressed as mean±SEM of percentage of total TgCAMα_{1B}AR binding (n=4 for each group). P=nonsignificant compared with TgCAMα_{1B}AR (Student *t* test) for each hybrid Tg mouse line.

CAMα_{1B}AR mice (Figure 1B and 1C). We also verified that GRK activity was not compromised in the hybrid Tg mice. In vitro GRK phosphorylation assays illustrate that phosphorylation activity was similar in single-Tg and hybrid Tg mice (data not shown). As shown in Figure 1D, myocardial α_1AR density in the hybrid CAMα_{1B}AR and the various GRK-overexpressing lines of Tg mice was equal to single-Tg CAMα_{1B}AR mouse myocardial α_1AR levels. The myocardial α_1AR density in the different GRK-overexpressing lines of Tg mice was equal to endogenous levels found in NLC mice (data not shown). The α_1AR overexpression seen in the wild-type (WT) α_{1B}AR was preserved in the presence of all second transgenes in the hybrid lines (data not shown).

In Vivo Inhibition of Hypertrophy

CAMα_{1B}AR mice have an elevated heart-to-body weight ratio compared with NLC mice (Figure 2), consistent with earlier findings that demonstrated that these mice had myocardial hypertrophy.[8] When the CAMα_{1B}AR mice were crossed with animals overexpressing GRK2 (3-fold or 20-fold), the heart-to-body weight ratio remained elevated (Figure 2). However, the presence of GRK3 overexpression in the CAMα_{1B}/GRK3

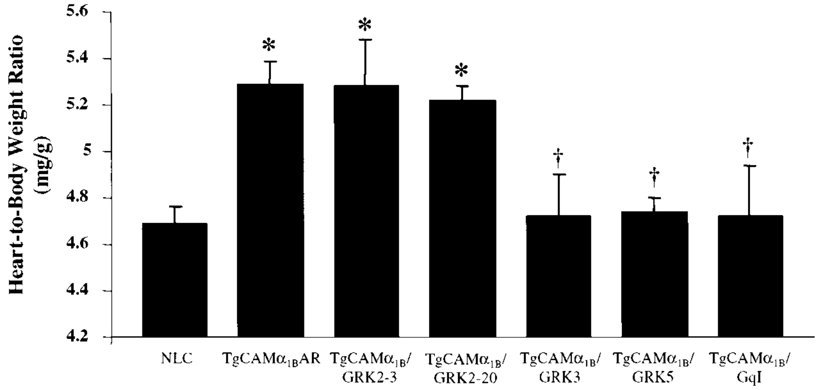

Figure 2. In vivo inhibition of CAMα_{1B}AR-induced hypertrophy. Hearts were excised, weighed, and normalized to total body weight and expressed as a mean±SEM of the (mg/g) ratio. Number of individual mice per group were as follows: NLC, n=13; TgCAMα_{1B}AR, n=13; TgCAMα1B/GRK2-3, n=10; TgCAMα_{1B}/GRK2-20, n=9; TgCAMα_{1B}/GRK3, n=8; TgCAMα_{1B}/GRK5, n=7; and TgCAMα_{1B}/GqI, n=5. *$P<0.05$ vs NLC, †$P<0.05$ versus TgCAMα_{1B}AR.

animals ablated CAMα_{1B}AR-induced hypertrophy, as did concomitant GRK5 overexpression (Figure 2). Thus, GRK3 and GRK5 overexpression were each capable of attenuating CAMα_{1B}AR-induced hypertrophy, whereas increased cardiac GRK2 activity had no effect.

To verify that myocardial hypertrophy seen in the CAMα_{1B}AR animals is the result of enhanced Gq signaling, we created hybrid Tg mice with CAMα_{1B}AR overexpression and overexpression of a peptide inhibitor of the receptor-Gq interface. This peptide (GqI) represents the last 54 amino acids of murine Gαq(305–359) and has been shown to specifically inhibit Gq signaling in vivo.[5] Similar to CAMα_{1B}/GRK3 mice, CAMα_{1B}/GqI animals had a heart-to-body weight ratio equal to that of NLCs, demonstrating that signaling through Gq is responsible for the CAMα_{1B}AR phenotype.

CAMα_{1B}AR Induced DAG Activation

Specific interactions between GRKs and α_{1B}ARs in vivo should result in attenuated signal transduction because of receptor desensitization, and this could account for the inhibition of CAMα_{1B}AR-induced hypertrophy by overexpression of GRK3 and GRK5. Signaling through α_{1B}AR-Gq proceeds through PLC activation, resulting in DAG accumulation. Therefore, basal DAG content was quantified in lipid fractions of hearts from Tg mice. Myocardial DAG content was significantly elevated 60% in CAMα_{1B}AR hearts (Figure 3), consistent with previous data.[8] A similar elevation in myocardial DAG content was observed in both CAMα_{1B}/GRK2-3 (3.87±0.31 pmol DAG/nmol lipid phosphate, n=4) and CAMα_{1B}/GRK2-20 (4.93±0.49 pmol DAG/nmol lipid phosphate [n=4] versus NLC 2.53±0.31 pmol DAG/nmol lipid phosphate [n=4]) (Figure 3). Thus, like hypertrophy, CAMα_{1B}AR signaling is not altered by GRK2 overexpression. In contrast to these results, myocardial DAG content was significantly lowered in hybrid CAMα_{1B}/GRK3 mice compared with CAMα_{1B}AR animals demonstrating that signaling through CAMα_{1B}ARs is attenuated by GRK3 overexpression (Figure 3).

Interestingly, and in contrast to the results seen in the heart-to-body weight ratios, hybrid CAMα_{1B}/GRK5 mice still had significantly elevated myocardial DAG content, similar to levels seen in CAMα_{1B}AR and CAMα_{1B}/GRK2 animals (Figure 3). To further study the interactions between GRK5 and α_{1B}ARs in vivo, we generated hybrid myocardium-targeted Tg mice overexpressing the WTα_{1B}AR and GRK5 (Table). As with CAMα_{1B}AR overexpression, WTα_{1B}AR overexpression in Tg mouse hearts leads to significant increases in myocardial DAG content compared with NLC animals (in pmol DAG/nmol lipid phosphate; NLC, 2.77±0.19 [n=4] versus WTα_{1B}AR, 4.18±0.28 [n=4]; $P<0.05$), consistent with previous results.[20] In hybrid WTα_{1B}/GRK5 mice, myocardial DAG content was still significantly elevated above NLC at a level similar to that of WTα_{1B}AR mice (4.98±0.54, n=6). Thus, it appears that although GRK5 expression was capable of inhibiting the development of α_{1B}-induced hypertrophy, it does not affect

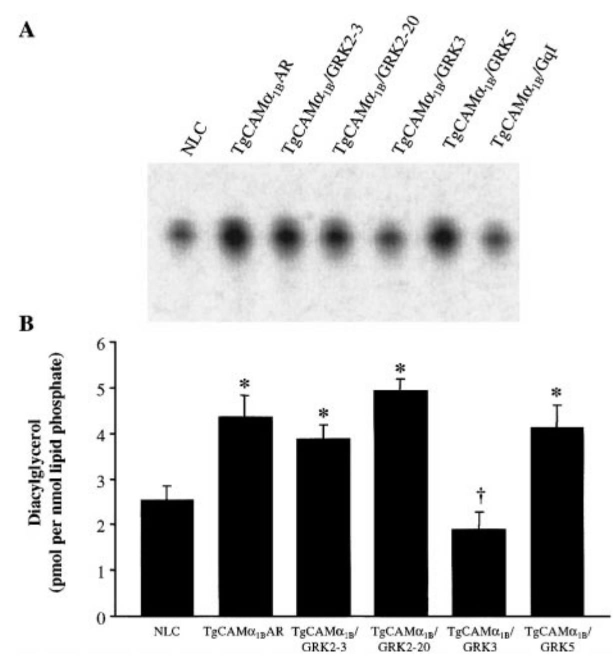

Figure 3. In vivo GRK influence on basal CAMα_{1B}AR-induced DAG content. Lipid extraction was performed on NLC, single-Tg, and hybrid Tg mouse hearts as described in Materials and Methods. DAG content was quantified using 50 nmol of lipid phosphate as described. A, Representative autoradiogram of thin-layer chromatography results from DAG assay in listed mice. B, Quantification of basal DAG activity expressed as mean±SEM. Number of mice examined per group was as follows: NLC, n=4; TgCAMα_{1B}AR, n=5; TgCAMα_{1B}/GRK2-3, n=5; TgCAMα_{1B}/GRK2-20, n=4; TgCAMα_{1B}/GRK3, n=5; and TgCAMα_{1B}/GRK5, n=6. *$P<0.05$ vs NLC, †$P<0.05$ vs TgCAMα_{1B}AR.

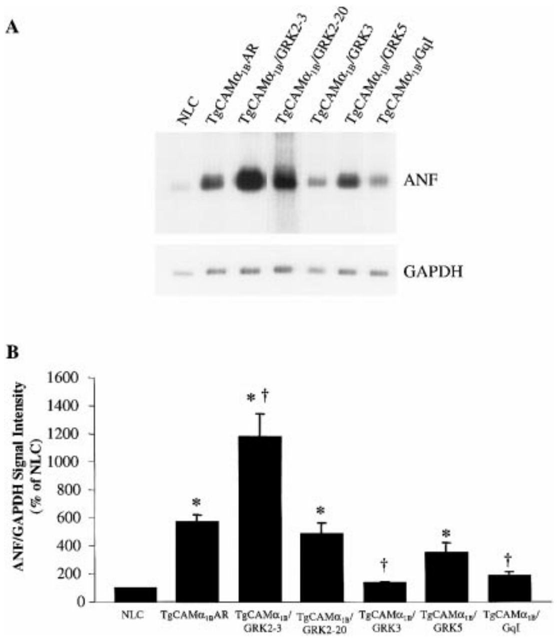

Figure 4. Ventricular ANF levels. A, Representative Northern blot of 10 μg of total RNA isolated from ventricles of NLC and Tg mouse hearts and probed with a mouse ANF cDNA (top panel). Blots were stripped and reprobed with a rat GAPDH cDNA (bottom panel). B, Quantification of ANF signal. Signals from the ANF blots were counted on a Molecular Dynamics PhosphorImager and normalized to GAPDH levels. GAPDH mRNA levels were similar in all ventricular samples from the different mice tested. Data shown are mean±SEM for the ANF signal corrected by GAPDH and expressed as a percentage of the ANF/GAPDH signal for NLC. Number of mice examined for each group was as follows: NLC, n=8; TgCAMα_{1B}AR, n=9; TgCAMα_{1B}/GRK2-3, n=4; TgCAMα_{1B}/GRK2-20, n=5; TgCAMα_{1B}/GRK3, n=4; TgCAMα_{1B}/GRK5, n=3; and TgCAMα_{1B}/GqI, n=4. **P*<0.05 vs NLC, †*P*<0.05 vs TgCAMα_{1B}AR.

basal α_{1B}AR/PLC signaling in the heart as examined in vivo after either WT or CAMα_{1B}AR overexpression.

Ventricular ANF mRNA

A central molecular characteristic of ventricular hypertrophy is the upregulation of a number of genes normally expressed in fetal myocardium. This includes ventricular expression of ANF. To investigate this in our series of hybrid Tg mice, we performed Northern blots on ventricular RNA, normalizing mouse ANF expression to an internal control, GAPDH (see Materials and Methods). Ventricles from NLC mice and the single GRK–overexpressing Tg mice demonstrated very faint or undetectable ANF mRNA expression, which was consistent with inactivation of this gene in normal adult ventricular myocardium (data not shown). We found that ventricular ANF mRNA levels were increased ≈700% in CAMα_{1B}AR mice (Figure 4), which is similar to previous findings.[8] Hybrid CAMα_{1B}/GRK2 ventricles still exhibited enhanced ANF mRNA levels that actually were, for reasons unknown, even higher than those of CAMα_{1B}AR mice for the TgGRK2-3, whereas CAMα_{1B}/GRK2-20 mice had ANF levels equivalent to those of CAMα_{1B}AR mice (Figure 4). Conversely, CAMα_{1B}/GRK3 animals had ventricular ANF mRNA levels equal to NLC values (Figure 4). This attenuation of ANF mRNA levels induced by CAMα_{1B}AR overexpression was also seen in hybrid CAMα_{1B}/GqI mice (Figure 4), demonstrating that Gq coupling is responsible for triggering this ANF response. Thus, as with hypertrophy and DAG signaling, GRK3 overexpression attenuates the CAMα_{1B}AR phenotype, whereas GRK2 overexpression does not significantly alter the CAM phenotype.

Interestingly, CAMα_{1B}/GRK5 mice exhibited lower ventricular ANF expression (350±70% of NLC, n=3) compared with that of CAMα_{1B}AR mice (570±50% of NLC, n=9), although this attenuation was not statistically significant (Figure 4). Moreover, the ANF mRNA levels were still significantly higher than levels seen in NLC mice, suggesting that GRK5 overexpression does not totally block CAMα_{1B}AR-induced ANF expression. To further examine the effects of GRK5 on α_{1B}AR-Gq–mediated ventricular ANF induction, we studied ANF expression in WTα_{1B}AR mice and WTα_{1B}/GRK5 animals. WTα_{1B}AR mice also exhibited elevated ventricular ANF mRNA levels (505±51% of NLC [n=5]; *P*<0.05). Interestingly, we have previously shown that this occurs without any myocardial hypertrophy.[20] Consistent with findings shown in Figure 4, hybrid WTα_{1B}/GRK5 mice also had significantly enhanced ventricular ANF mRNA (492±53% of NLC [n=5]; *P*<0.05), demonstrating that GRK5 does not eliminate ANF expression after enhanced α_{1B}AR-Gq signaling. WTα_{1B}/GRK2-3 also had maintained elevated ANF expression (555±49% of NLC [n=3]; *P*<0.05), suggesting that regulation of α_{1B}AR by GRKs is similar for both the WT and CAMα_{1B}AR and that GRK2 does not affect α_{1B}AR signaling in the heart.

ERK and JNK Activity in Response to α_1AR Activation and GRK Expression

Because the data presented above demonstrate that there is apparent specificity among GRK2, GRK3, and GRK5 in desensitizing α_{1B}ARs in vivo in the heart, we examined the effects of these 3 GRKs on endogenous myocardial α_1AR signaling. A relevant signaling pathway that has been demonstrated to be activated by α_1AR stimulation in the heart is the MAPK pathway, including ERK1/ERK2 and JNK1.[23–25] In this study, we used ERK and JNK activity assays to determine whether endogenous in vivo myocardial α_1AR-MAPK signaling is altered in GRK Tg mice. Mice were injected intraperitoneally with either saline (basal signaling) or the α_1-agonist PE, and ERK and JNK activity induced by PE (over basal activity) was determined in the GRK Tg animals and compared with values in NLC mice. After 10 minutes, injected mice were euthanized, their hearts extracted and homogenized, and ERK and JNK were immunoprecipitated for an in vitro kinase assay. Basal ERK and JNK activity measured after saline injection was similar or equivalent in NLC and single GRK–overexpressing mice (data not shown). In NLC mice, PE induced a significant 40% increase in ERK activity over basal levels of activity (Figure 5A). As shown in Figure 5A, GRK2 overexpression in either the GRK2-3 or GRK2-20 animals did not inhibit myocardial PE-induced ERK activity, whereas GRK3 or GRK5 overexpression significantly attenuated PE-induced ERK activity. Similar to the ERK activity results, JNK activity was attenuated in mice overexpressing GRK3, whereas GRK2 overexpression at either low or high amounts had no impact on JNK signaling

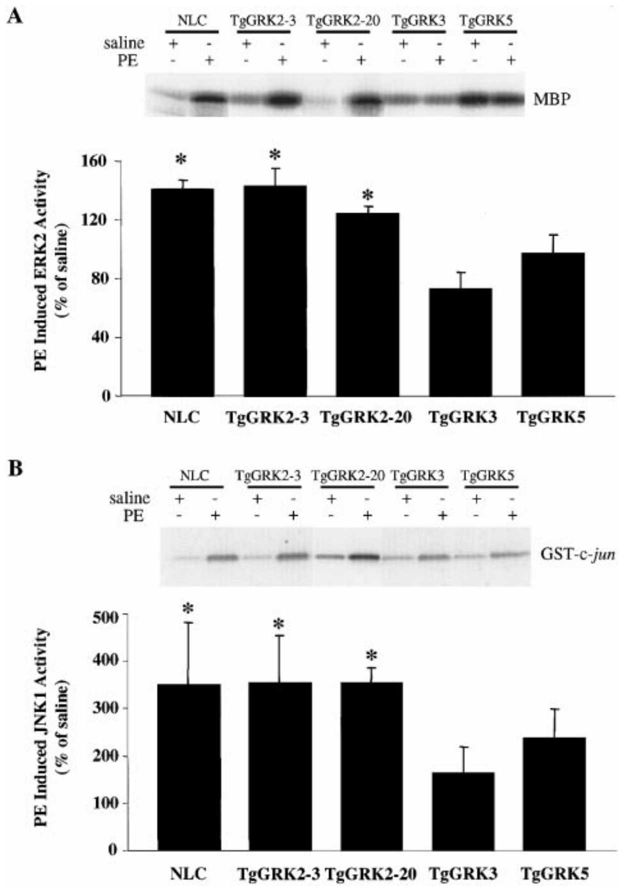

Figure 5. ERK2 activation in response to the α_1AR agonist PE. Mice were given intraperitoneal injections of 200 μL of saline or 500 mmol/L PE and allowed to recover for 10 minutes. ERK2 was immunoprecipitated from heart extracts, and kinase activity was measured using MBP as a substrate for phosphorylation assays. A, Representative autoradiogram of the [γ-^{32}P]ATP phosphorylated form of MBP due to ERK2 kinase activity in NLC or single-Tg mice injected with saline or PE and quantification of ERK2 activity expressed as level of stimulation with PE over level of stimulation in saline-injected mice (% of saline). Data are mean$\pm$SEM for number of mice as follows: NLC, n=9; TgGRK2-3, n=6; TgGRK2-20, n=4; TgGRK3, n=5; and TgGRK5, n=7. B, Representative autoradiogram of the [γ-^{32}P]ATP-phosphorylated form of glutathione *S*-transferase (GST)–c-jun due to JNK1 activity in NLC or single-Tg mice injected with saline or PE and quantification of JNK1 activity expressed as level of stimulation with PE over level of stimulation in saline-injected mice (% of saline). Data are mean$\pm$SEM for number of mice as follows: NLC, n=5; TgGRK2-3, n=5; TgGRK2-20, n=5; TgGRK3, n=5; and TgGRK5, n=4. **P*<0.05 vs NLC induction with PE as compared with saline injection.

induced by the α_1AR agonist PE (Figure 5B). Similar to what was seen with ANF signaling (Figure 4), GRK5 overexpression attenuated JNK signaling only partially as compared with NLC activation (Figure 5B). These data further suggest that GRK3 is the primary GRK responsible for desensitizing in vivo α_1AR signaling in the heart. GRK5 had variable effects on α_{1B}AR signaling, and the complexity of GRK5 regulation of in vivo α_{1B}AR signaling remains to be fully elucidated.

Discussion

The results of this study, which uses 15 distinct Tg mouse lines, reveal 2 significant findings. The first is the demonstration of the feasibility and power of using hybrid Tg mice with myocardial overexpression of G protein–coupled receptors with concomitant GRK overexpression to specifically study their in vivo interactions. Secondly, this hybrid Tg strategy revealed that myocardial α_1ARs are in vivo targets for GRK3-mediated desensitization but not for GRK2, the GRK most abundantly expressed in the myocardium. These differing effects of GRK2 and GRK3 were confirmed in hybrid Tg mice overexpressing either the WT α_{1B}AR or a CAM α_{1B}AR, as well as in studies designed to examine endogenous myocardial α_1AR signaling. The effects of these GRKs were assessed on the hypertrophic phenotype of these mice, Gq-PLC signaling (via myocardial DAG content), and endogenous α_1AR-MAPK signaling in the heart. In addition to the differing results found with these 2 GRKs, overexpression of GRK5 caused variable effects on in vivo α_{1B}AR signaling. GRK5 was capable of attenuating endogenous α_1AR-mediated ERK activity and CAMα_{1B}AR-induced hypertrophy. However, it had a lesser effect on CAM and WTα_{1B}AR-mediated DAG levels, ventricular ANF mRNA expression, and JNK activity.

The hearts of these hybrid Tg mice were used in this study as novel "in vivo reaction vessels," making it possible to study the biochemical and physiological consequences of the actions of one transgene product (ie, GRK) on another (ie, α_{1B}AR). This study has demonstrated the power of this simple crossbreeding strategy that can be used to address questions regarding GRK specificity on other receptors or for dissecting individual phenotypes of other Tg models. Importantly in this study, expression levels of α_{1B}ARs and individual GRKs driven by the same αMyHC promoter, when expressed concomitantly in the hybrid mice, did not differ from the levels of overexpression seen in the individual Tg parental lines. This is an important finding, given that promoter competition might be expected to occur, limiting the overexpression of 1 or both of the transgenes, and thus limiting the usefulness of this strategy. Furthermore, our previous studies of Tg mice using the αMyHC promoter have demonstrated that transgene expression was homogenous in nature throughout the heart[8,16]; thus, both transgenes should be expressed in a similar fashion when together.

Using these hybrid Tg mice in this study allowed us to investigate the in vivo specificity of GRK2 (also known as βARK1), GRK3 (βARK2), and GRK5 on α_{1B}ARs in the heart. This is an area of study concerning the 6-member GRK family, for which definitive information has been lacking. A majority of in vitro experiments using heterologous cell culture expression systems with a variety of overexpressed G protein–coupled receptors have shown limited substrate selectivity for these 3 ubiquitously expressed GRKs.[10–14] Importantly in the present study, we have revealed that these 3 GRKs differ in their ability to desensitize α_1ARs in vivo in the heart. Our study differs from previous in vitro findings regarding the α_{1B}AR using heterologous cell culture expression systems, in which overexpression of either GRK2 or GRK3 promoted α_{1B}AR desensitization via agonist-induced phosphorylation.[12] Our results demonstrate that GRK2 is ineffective in regulating either the overexpressed CAMα_{1B}AR or the WTα_{1B}AR, and more importantly, signaling through endogenous myocardial α_1ARs (of which the predominant subtype in the mouse heart is the α_{1B}[1]) was also unaltered by GRK2 overexpression. Interestingly, signaling through each type of α1BAR in the different hybrid Tg mice was significantly attenuated by GRK3 overexpression demonstrating an in vivo selectivity between GRK2 and GRK3 in the heart. Similar to Diviani et al,[12] we found variable effects of GRK5 on agonist-mediated desensitization of myocardial α_{1B}ARs. Importantly, levels of GRK overexpression, as compared with total endogenous NLC GRK activity, between the different Tg mice could not explain the lack of GRK2 effect, given that neither ≈3-fold GRK2 overexpression nor ≈20-fold GRK2 overexpression was sufficient to attenuate in vivo α_{1B}AR signaling, whereas ≈5-fold GRK3 overexpression (≈12-fold overexpression when compared with endogenous GRK3 levels) effectively abrogated all examined forms of α_{1B}AR signaling and eliminated the myocardial hypertrophy induced by CAMα_{1B}AR expression. Recently, it was shown by Iacovelli et al[15] in FRTL-5 cells stably overexpressing GRK2 that endogenous signaling through α_{1B}AR was not attenuated, whereas signaling through endogenous thyrotropin receptors was. Thus, there are now data to suggest that even in certain cell types in vitro, GRK2 does not desensitize the α_{1B}AR, which is consistent with our in vivo findings in the heart.

Despite their initial characterization as highly homologous isozymes, a pattern of in vivo differences between GRK2 and GRK3 is now emerging. In addition to our current findings concerning α_{1B}AR signaling, we have demonstrated previously that GRK3 does not, whereas GRK2 does, regulate endogenous βAR signaling in the heart.[18] As is the case with α_{1B}AR signaling, these in vivo βAR findings differ from in vitro results that demonstrate that both GRK2 and GRK3 phosphorylate and desensitize β_1ARs, the primary βAR subtype expressed in the myocardium.[11] Furthermore, studies with TgGRK3 animals reveal GRK specificity for the endogenous myocardial thrombin receptor.[18] The thrombin receptor is one receptor in which a distinct difference in GRK-mediated desensitization has been shown to exist in vitro between GRK2 and GRK3.[26] Differences in the in vivo myocardial regulation of angiotensin II receptors have also been seen in TgGRK3 and TgGRK2 mice.[17,18] Thus, the use of Tg technology has begun to clarify GRK specificity in the in vivo heart. Collectively, these data suggest that GRK2 and GRK3 have distinct substrates in the intact heart and do not exhibit redundancy in the normal regulation of myocardial function.

The regulation of GRK2 and GRK3 may provide insight into why these highly homologous kinases differ in their in vivo substrate selectivity in the heart. Both GRK2 and GRK3 are cytosolic enzymes that undergo a membrane-targeting event before their phosphorylation of agonist-occupied receptors.[9,10] For these 2 GRKs, this is accomplished by a specific protein-protein interaction between the carboxyl-terminal domain of GRK2 and GRK3 and $G_{\beta\gamma}$ subunits released from activated heterotrimeric G proteins.[27,28] Interestingly, the most divergent region between GRK2 and GRK3 is within the mapped $G_{\beta\gamma}$ binding domain.[28–30] This may indicate differential affinities of these 2 GRKs for $G_{\beta\gamma}$ subunits, and the availability of G$\beta\gamma$s may be influenced by cell type and by the G protein–coupled receptor activated. Thus, $G_{\beta\gamma}$s released after myocardial α_{1B}AR stimulation may bind to GRK3 with higher affinity than that for GRK2, and vice versa for $G_{\beta\gamma}$s released by β_1ARs in vivo. Importantly, in vitro evidence supports the notion that GRK2 and GRK3 can be targeted to membranes in a receptor- and $G_{\beta\gamma}$-dependent manner.[31]

Although the in vivo G protein–coupled receptor specificity for GRK2 and GRK3 is becoming clearer, the understanding of in vivo GRK5 actions in the heart are more complex. It has been shown that in contrast to GRK2 overexpression, myocardial GRK5 overexpression, like that of GRK3, does not alter in vivo angiotensin II signaling.[17] However, like GRK2 overexpression, βAR signaling in vivo was significantly attenuated in the hearts of TgGRK5 mice.[17] The present results reveal conflicting findings regarding the in vivo actions of GRK5 on myocardial α_{1B}AR signaling. Whereas in vivo GRK5 attenuated CAMα_{1B}AR-induced hypertrophy and endogenous α_{1B}AR agonist-stimulated ERK2 activity, GRK5 was incapable of inhibiting overexpressed CAMα_{1B}AR- or WT α_{1B}AR-induced DAG and TgWTα_{1B}AR-induced ANF levels, and only partially attenuated ANF levels in the TgCAMα_{1B}AR hearts and JNK1 activity in the hearts expressing endogenous α_{1B}ARs. These results suggest that DAG signaling due to α_{1B}AR stimulation does not result in hypertrophy and that ventricular ANF expression, although concomitant with it, is not sufficient for hypertrophy. In fact, recent studies associate ANF with inhibition of proliferation of nonmyocardial cells and antihypertrophic effects in cardiomyocytes.[32–35] In contrast, it appears that MAPK activation in response to α_{1B}AR-Gq stimulation may be important for hypertrophy. In fact, these findings may shed light on the specific signaling pathways responsible for the progression of α_{1B}AR activation to myocardial hypertrophy, although this remains to be determined.

G protein–coupled receptors play integral roles in cardiac function, and examination of their regulation by GRKs is important for understanding cardiac homeostasis and the regulation of compensation during disease states. For example, heart failure is associated with a constellation of changes, including increases in circulating catecholamines, decreases in βAR density, and increases in GRK2.[36] Additionally, α_1AR density has been shown to be elevated when heart failure develops.[37] Therefore, in disease states when βARs are downregulated, α_1ARs become a more predominant population of the myocardial ARs. Importantly, as the findings regarding in vivo GRK substrate specificity in this report reveal, potentially increased α_1AR signaling in the compromised heart would be insensitive to the elevated GRK2 levels associated with heart disease. This may provide a mechanism that attempts to maintain cardiac output in response to catecholamines. This lack of GRK2 effect on α_1ARs could lead to enhanced Gq signaling responsible for the initial adaptive hypertrophy response in the compromised heart. Testing these hypotheses will be the subject of future studies.

In summary, results from this study definitively demonstrate that GRK3 desensitizes α_{1B}AR-mediated signaling in vivo, whereas the highly homologous GRK2 had no effect on signaling through this G protein–coupled receptor. These results illustrate that although in vitro studies set the foundation for understanding receptor-kinase interactions, in vivo studies are required to fully elucidate in vivo selectivity. Furthermore, it is becoming clear from Tg studies that GRK2, GRK3, and GRK5 play distinct roles in the normal regulation of myocardial signaling and function.

Acknowledgments

This work was supported in part by National Institutes of Health Grants HL-16037 (to R.J.L.), HL-61690 and HL-59533 (to W.J.K.), GM44944 (to J.L.B.) and by a Grant-in-Aid from the North Carolina Affiliate of the American Heart Association (to W.J.K.). R.J.L. is an investigator of the Howard Hughes Medical Institute. J.L.B. is an Established Investigator of the American Heart Association.

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