

# Receptor-specific *in vivo* desensitization by the G protein-coupled receptor kinase-5 in transgenic mice

(myocardial contractility/ $\beta$ -adrenergic receptor kinase/angiotensin II receptor/adenylylase)

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**ABSTRACT** Transgenic mice were generated with cardiac-specific overexpression of the G protein-coupled receptor kinase-5 (GRK5), a serine/threonine kinase most abundantly expressed in the heart compared with other tissues. Animals overexpressing GRK5 showed marked  $\beta$ -adrenergic receptor desensitization in both the anesthetized and conscious state compared with nontransgenic control mice, while the contractile response to angiotensin II receptor stimulation was unchanged. In contrast, the angiotensin II-induced rise in contractility was significantly attenuated in transgenic mice overexpressing the  $\beta$ -adrenergic receptor kinase-1, another member of the GRK family. These data suggest that myocardial overexpression of GRK5 results in selective uncoupling of G protein-coupled receptors and demonstrate that receptor specificity of the GRKs may be important in determining the physiological phenotype.

A diverse family of receptors activate G protein-coupled signal transduction pathways to elicit multiple biologic functions. Two G protein-coupled receptors that are closely involved in the regulation of cardiac function and growth in response to neurohormonal and mechanical stimuli are the  $\beta_1$ -adrenergic receptor ( $\beta_1$ -AR) and the angiotensin II (AngII) receptor (1, 2). These receptors, when activated by agonists, stimulate the heterotrimeric G proteins Gs and Gq, respectively, leading to the intracellular accumulation of second messengers; notably, cAMP from  $\beta_1$ -AR-Gs coupling and inositol trisphosphate and diacylglycerol from AngII-Gq coupling (3, 4). With persistent stimulation by agonist, many G protein signal transduction systems demonstrate diminished responsiveness referred to as desensitization. Two classes of serine/threonine kinases can regulate receptors through rapid receptor phosphorylation; the second messenger-activated protein kinases, such as the cAMP-dependent protein kinase and protein kinase C (5), and the G protein-coupled receptor kinases (GRKs), which phosphorylate activated receptors, leading to homologous desensitization (5). Currently there are six known members of the emerging GRK family, of which GRK2 (commonly known as  $\beta$ ARK1), GRK3, GRK5, and GRK6 are expressed in the heart (6, 7). Previous studies have shown that  $\beta$ ARK1 is a critical *in vivo* regulator of cardiac function (8), and it is elevated in chronic human heart failure (9). GRK5, as shown by Northern analysis, is most abundantly expressed in the heart compared with other tissues (10), although its importance in myocardial  $\beta$ -AR signaling has not been established.

Several unique features distinguish agonist-induced desensitization mediated by these two GRKs ( $\beta$ ARK1 and GRK5). The primarily cytosolic  $\beta$ ARK1 requires a membrane-targeting or translocation event before phosphorylating agonist-occupied receptors, which occurs via a direct physical inter-

action between residues within  $\beta$ ARK's carboxyl terminus and the dissociated, membrane-anchored  $\beta\gamma$  subunits of G proteins (11, 12). Unlike  $\beta$ ARK1, GRK5 does not undergo agonist-dependent translocation from cytosol to membrane, but rather is constitutively membrane-bound (10). Previous *in vitro* reconstitution and intact cell experiments have demonstrated agonist-dependent phosphorylation of many G protein-coupled receptors by various GRKs, including  $\beta_1$ -ARs, the predominant subtype in the heart (13–15). Since the myocardial  $\beta$ -AR system is so critical in normal and pathophysiological states (8, 9), we sought to determine the extent and specificity to which targeted cardiac overexpression of GRK5 would desensitize G protein-coupled receptors *in vivo*. Furthermore, since myocardial function is greatly influenced by anesthetic agents, studies were also performed in chronically instrumented transgenic mice in the awake, conscious state.

## METHODS

**Generation and Identification of Transgenic Mice.** Transgenic mice were created using the  $\alpha$ -myosin heavy chain promoter to direct cardiac-specific overexpression of the entire coding region for bovine GRK5. For construction of the GRK5 transgene, the 1.8-kb *EcoRI*-*XbaI* open reading frame was isolated from a previous constructed plasmid (10) and ligated into a plasmid containing the SV-40 intron poly(A) signal (8) to yield pGEM-GRK5-SV-40. This construct was then digested with *EcoRI*, blunted with Klenow DNA polymerase and subsequently digested with *SacI*. A *SacI*-blunt 5.5-kb  $\alpha$ -myosin heavy chain promoter (16) cassette was then ligated into this plasmid to generate the plasmid pGEM- $\alpha$ MHC-GRK5-SV-40. The transgene was then linearized and purified before pronuclear injections done by the Duke Comprehensive Cancer Center Transgenic Facility (8). Two lines were established, TG GRK5-25 and TG GRK5-45. Litter sizes and postneonatal development was indistinguishable from nontransgenic littermate controls. Offspring were screened by Southern blot analysis with a probe to the SV-40 sequences. Second generation adult animals, 2–5 months of age from the TG GRK5-45 line were used for all physiological studies. Institutional Review Board approval for all mouse experiments was obtained from the University of California at San Diego and from Duke University.

**Western Analysis.** Immunodetection of myocardial levels of GRK5 in control nontransgenic and transgenic mice was performed in solubilized myocardial membranes followed by

Abbreviations:  $\beta_1$ -AR,  $\beta_1$ -adrenergic receptor; AngII, angiotensin II; GRK, G protein-coupled receptor kinase; LV, left ventricle; L-NAME, *N*-nitro-L-arginine methyl ester; AT<sub>1</sub>, angiotensin II type 1 receptor.

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immunoprecipitation. Myocardial extracts were prepared by homogenization of excised hearts in ice-cold lysis buffer (2 ml of 25 mM Tris-Cl, pH 7.5/5 mM EDTA/5 mM EGTA/10  $\mu$ g of leupeptin per ml/20  $\mu$ g of aprotinin per ml/1 mM phenylmethylsulfonyl fluoride) and centrifuged at 48,000  $\times$  *g* for 30 min. The supernatants that contain soluble kinases (i.e.,  $\beta$ ARK1 and overexpressed GRK5) were discarded and the membranes rehomogenized in ice-cold solubilization buffer (2 ml of 50 mM Tris-Cl, pH 8.0/150 mM NaCl/5 mM EDTA/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS/10 mM NaF/10 mM sodium pyrophosphate/0.1 mM phenylmethylsulfonyl fluoride) and clarified by centrifugation. GRK5 was immunoprecipitated from 1 ml of clarified extract with 1:500 (2  $\mu$ l) anti-GRK5 serum (10) and 35  $\mu$ l of a 50% slurry of protein A-agarose conjugate agitated for 1 hr at 4°C. Immune complexes were washed five times with ice-cold solubilization buffer, and the dried agarose beads were resuspended in 30  $\mu$ l of protein-gel loading buffer and electrophoresed through SDS/12% polyacrylamide gels and transferred to nitrocellulose. The endogenously and exogenously overexpressed  $\approx$ 68-kDa GRK5 protein was visualized with a monoclonal antibody raised against an epitope within the carboxyl terminus of bovine GRK5, and detection of anti-mouse IgG conjugated with horseradish peroxidase was carried out by enhanced chemiluminescence (ECL; Amersham).

**GRK Activity in Rhodopsin Phosphorylation Assays.** Hearts were homogenized as described above. The first supernatant was removed, and the pelleted membranes were rehomogenized in lysis buffer containing 250 mM NaCl, to dissociate membrane-bound kinase (i.e., GRK5), and centrifuged at 48,000  $\times$  *g* for 30 min. The membrane protein supernatant was further purified by adding a slurry of 50% (vol/vol) diethylaminoethyl Sephacel (pH 7.0) in the presence of 250 mM NaCl, incubated on ice for 30 min, and run over a small disposable column. Final supernatants were eluted with no salt buffer and concentrated using a Centricon (Amicon) microconcentrator. Protein concentration was determined by Bradford assay (17). Concentrated membrane extracts (50  $\mu$ g of protein) were incubated with rhodopsin-enriched rod outer segments (8) in lysis buffer (75  $\mu$ l) with 10 mM MgCl<sub>2</sub> and 0.1 mM ATP (containing [ $\gamma$ -<sup>32</sup>P]ATP). After incubating in white light for 15 min at room temperature, reactions were quenched with ice cold lysis buffer (300  $\mu$ l) and centrifuged for 15 min at 13,000  $\times$  *g*. Sedimented proteins were resuspended in 25  $\mu$ l of protein-gel loading dye and electrophoresed through SDS/12% polyacrylamide gels (8). Phosphorylated rhodopsin was visualized by autoradiography of dried polyacrylamide gels and quantified using a PhosphorImager (Molecular Dynamics).

**Adenylyl Cyclase Activity.** Myocardial sarcolemmal membranes were prepared by homogenizing whole hearts in ice-cold cyclase buffer (50 mM Hepes, pH 7.3/150 mM KCl/5 mM EDTA). Nuclei and tissue were separated by centrifugation at 800  $\times$  *g* for 10 min, and the crude supernatant was then centrifuged at 20,000  $\times$  *g* for 10 min. Sedimented proteins were resuspended at a concentration of 2–3 mg of protein per ml in assay buffer (50 mM Hepes, pH 7.3/5 mM MgCl<sub>2</sub>). Membranes (20–30  $\mu$ g of protein) were incubated for 15 min at 37°C under basal conditions and in the presence of isoproterenol or NaF and cAMP was quantitated by standard methods described previously (8).

**Physiological Evaluation. Hemodynamic evaluation in anesthetized mice.** Mice were anesthetized with a mixture of ketamine and xylazine (8, 18). After endotracheal intubation, mice were connected to a rodent ventilator. Either carotid artery was cannulated with a flame-stretched PE-50 catheter connected to a modified P-50 Statham transducer. Following bilateral vagotomy, the chest was opened, and either a 1.8-French or 2-French high-fidelity micromanometer catheter was inserted into the left atrium, advanced through the mitral valve, and secured in the left ventricle (LV). Hemodynamic

measurements were recorded at baseline and 45–60 sec after injection of incremental doses of isoproterenol or AngII. Doses of isoproterenol were specifically chosen to maximize the contractile response but limit the increase in heart rate. Continuous high-fidelity LV and fluid-filled aortic pressure was recorded simultaneously at baseline and 45–60 sec after each dose of agonist on an eight-channel chart recorder and in digitized form at 2000 Hz for later analysis. Experiments were then terminated with an overdose of pentobarbital. Hearts were rapidly excised, and individual chambers were separated, weighed, and then frozen in liquid N<sub>2</sub> for later analysis. Parameters measured were heart rate, aortic pressure, LV systolic and end diastolic pressure, and the maximal and minimal first derivative of LV pressure (LV  $dP/dt_{max}$ ,  $dP/dt_{min}$ ). Ten sequential beats were averaged for each measurement. The TG GRK5-45 and TG  $\beta$ ARK12 (8) lines were used for all *in vivo* physiological studies of  $\beta$ -AR and AngII receptor function.

**Alterations in loading conditions.** To determine the effect of acute changes in loading conditions on contractile function, LV systolic pressure was acutely increased in control mice (*n* = 4) with administration of the nitric oxide synthase inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME). Following instrumentation, hemodynamic measurements were continuously recorded under basal conditions and after progressive intravenous doses (0.025–2.5 mg) of L-NAME and analyzed as above.

**Hemodynamics in conscious mice.** For measurement of hemodynamic parameters in the conscious state, mice were anesthetized with the same mixture of ketamine and xylazine and were allowed to breathe spontaneously. A 1.8-French high-fidelity micromanometer catheter was inserted into the right carotid and advanced retrograde into the LV. The catheter was secured in the neck and tunneled to the back and exteriorized. Mice were allowed to recover from anesthesia (1.5–2 hr), and hemodynamic measurements were recorded in the quiet resting awake state. Hemodynamic parameters were analyzed as above.

**Statistical Analysis.** Data are expressed as mean values  $\pm$  SEM. To examine the effect of the various agonists on changes in hemodynamic parameters between the control and TG GRK5-45 groups, a two-way repeated measures (isoproterenol, AngII) or a one factor (L-NAME) analysis of variance (ANOVA) was used. Post hoc analysis with regard to differences in mean values between the groups at a specific dose were conducted with a Newman-Keuls test. To test for statistical significance of physiological parameters in conscious mice following recovery from anesthesia a two-way repeated measures ANOVA for two comparisons was used with a Newman-Keuls post hoc analysis. A Student's *t* test was used to test for statistical difference in adenylyl cyclase activities. For all analyses, *P* < 0.05 was considered significant.

## RESULTS

The extent of overexpression of GRK5 mRNA for the two lines of transgenic mice was first determined by Northern analysis (data not shown). Membrane-bound GRK5 protein was identified in control nontransgenic and GRK5 overexpressing animals via solubilization of cardiac membranes and immunoprecipitation of GRK5 followed by visualization by Western blotting (Fig. 1A). Increased GRK5 protein in the heart resulted in both lines of transgenic mice (Fig. 1A). To quantify the activity of membrane-bound GRK in transgenic mice, the membrane fractions from heart extracts were assayed for ability to catalyze the phosphorylation of the G protein-coupled receptor rhodopsin. Consistent with the marked overexpression of GRK5 protein, there was a marked increase in kinase activity in the membrane fraction of extracts from TG GRK5-45 (Fig. 1B). This kinase activity in the membrane fraction is presumed to be primarily GRK5, since the addition of purified  $\beta$  subunit of G protein, which activates  $\beta$ ARK1,

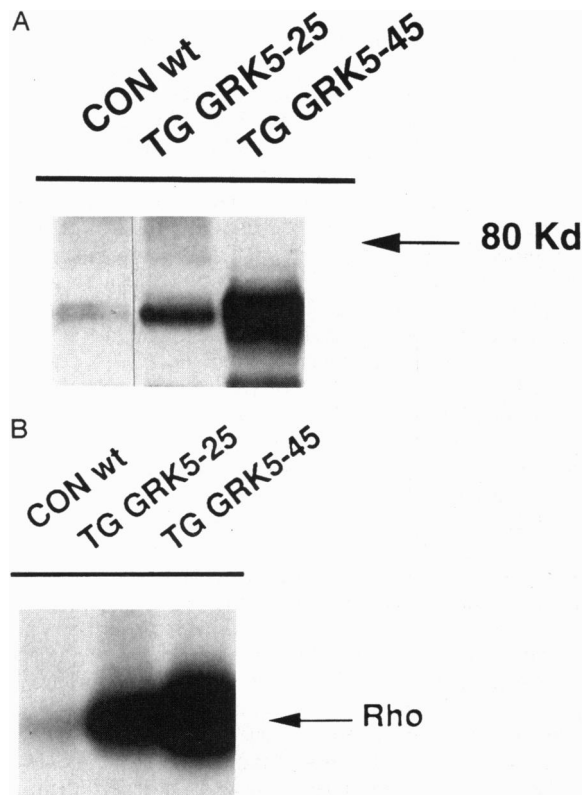


FIG. 1. Assessment of GRK5 expression and activity. (A) Immunodetection of myocardial levels of GRK5 in membrane extracts from control nontransgenic and transgenic mice. The endogenous and exogenously overexpressed 68-kDa GRK5 protein was visualized via Western blotting and chemiluminescence. The 80-kDa marker is shown. (B) Assessment of GRK activity in the membrane fraction of extracts from control and transgenic hearts using a rhodopsin phosphorylation assay. Shown is a representative autoradiograph of a dried gel where phosphorylated rhodopsin (Rho) is visualized.

did not lead to enhanced activity (data not shown). From these data, it was found that the TG GRK5-45 line had >30-fold overexpression of myocardial GRK5 compared with the levels of this kinase found in the hearts of nontransgenic control mice.

To examine the biochemical effects of this marked GRK5 overexpression on the myocardial  $\beta$ -AR system, we assessed receptor-effector coupling in sarcolemmal membranes from control and transgenic mouse hearts by measuring adenylyl cyclase activity under basal conditions and after maximal doses of isoproterenol and NaF (Table 1). In cardiac membranes from TG GRK5-45 mice, basal adenylyl cyclase activity as well as isoproterenol-stimulated activity, was significantly lower (Table 1). NaF-stimulated values were slightly lower in the TG GRK5-45 mice but did not reach statistical significance (Table 1). In addition, the density of myocardial  $\beta$ -ARs was found to be unchanged in the hearts of TG GRK5-45 animals (data not shown). Thus, there appears to be functional uncoupling of myocardial  $\beta$ -ARs due to the overexpression of GRK5, which

Table 1. Adenylyl cyclase activity in control and TG GRK5-45 hearts

Hearts	Activity, pmol/min per mg		
	Basal	ISO, $10^{-4}$ M	NaF, $10^{-3}$ M
Control, $n = 5$	$45.1 \pm 6.4$	$102.8 \pm 17.2$	$364.5 \pm 43.0$
TG GRK5-45, $n = 8$	$21.2 \pm 4.1^*$	$31.8 \pm 5.5^*$	$285.3 \pm 37.5$

ISO, isoproterenol.  
\* $P < 0.05$  compared to control.

indicates that, like  $\beta$ ARK1 (8), GRK5 can desensitize myocardial  $\beta$ -ARs.

To directly assess whether overexpression of GRK5 uncouples  $\beta$ -ARs, cardiac catheterization was used to measure catecholamine responsiveness *in vivo* in the intact anesthetized mouse (Fig. 2). Continuous measurements of heart rate and LV pressures were recorded before and after progressive doses of isoproterenol. At baseline, reduced LV systolic pressure and slower LV relaxation was observed in the TG GRK5-45 mice compared with control animals (Fig. 2 B and C). Marked blunting of the isoproterenol-induced inotropic response (Fig. 2A), as well as an attenuated fall in LV  $dP/dt_{min}$ , was seen with  $\beta$ -AR stimulation (Fig. 2B). The rise in LV systolic pressure was blunted compared with littermate controls (Fig. 2C) without a change in heart rate (Fig. 2D). LV end diastolic pressure was not statistically different between groups at baseline (control  $4.3 \pm 0.4$  versus transgenic  $2.9 \pm 0.5$  mmHg; 1 mmHg = 133 Pa) or with isoproterenol (control  $8.0 \pm 0.6$  versus transgenic  $6.0 \pm 0.8$  mmHg for the maximal dose). These data suggest that *in vivo*, myocardial  $\beta$ -ARs are targets for the membrane-bound GRK5 induced receptor phosphorylation and desensitization.

Because baseline contractility and responsiveness to sympathetic stimulation can be profoundly altered by general anesthesia (19, 20), hemodynamic evaluation was performed in chronically instrumented mice in the fully awake resting state. In control mice, recovery from anesthesia was associated with a significant increase in LV  $dP/dt_{max}$ , LV  $dP/dt_{min}$ , and heart rate (Table 2). In contrast, enhancement of contractile function with recovery from anesthesia was significantly blunted in the TG GRK5-45 mice (Table 2). Heart rate was similar in both

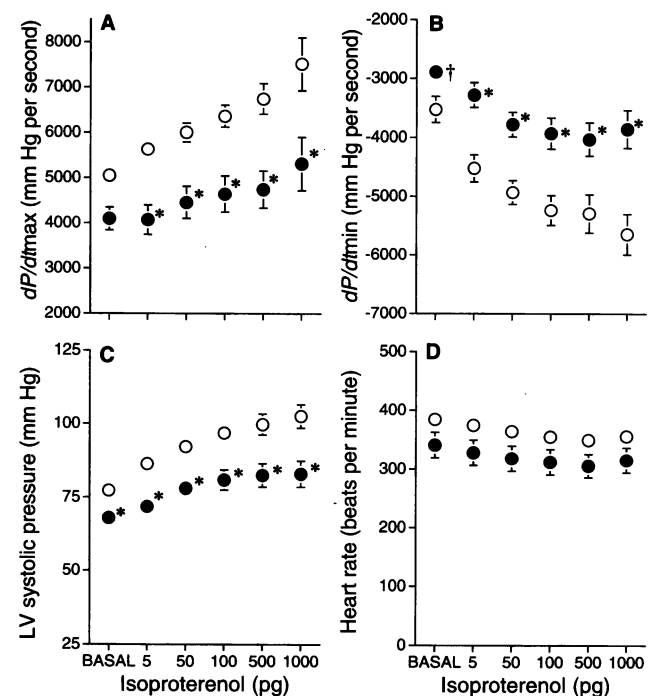


FIG. 2. *In vivo* assessment of LV contractile function of TG GRK5-45 ( $n = 10$ ) (●) and nontransgenic littermate control mice ( $n = 13$ ) (○). Cardiac catheterization was performed in intact anesthetized animals. Four measured parameters are shown at baseline and after progressive doses of isoproterenol. (A) LV  $dP/dt_{max}$ ; (B) LV  $dP/dt_{min}$ ; (C) LV systolic pressure; and (D) heart rate. Data were analyzed with a two-way repeated ANOVA. \*,  $P < 0.001$ ; and †,  $P < 0.05$ ; control versus transgenic. A significant between-group main effect in response to isoproterenol was found for (A)  $dP/dt_{max}$  ( $P < 0.0005$ ); (B)  $dP/dt_{min}$  ( $P < 0.001$ ); and (C) LV systolic pressure ( $P < 0.001$ ). The pattern of change between groups was statistically different for (B)  $dP/dt_{min}$ ,  $P < 0.05$ .

groups (Table 2). These results demonstrate that marked  $\beta$ -AR uncoupling and desensitization with GRK5 overexpression can significantly depress contractile function in mice with an integrated autonomic nervous system.

To determine whether GRK5 overexpression uncouples other G protein-coupled receptors, *in vivo* hemodynamic assessment was used to measure the physiologic response to AngII (Fig. 3). A sharp rise in LV systolic pressure in both transgenic mice and negative littermates resulted in response to infusion of progressive doses of AngII documenting the potent vasoconstrictor action of this neurohormone (Fig. 3C). Associated with the peripheral hemodynamic action of AngII, the effect on cardiac function included an increase in LV  $dP/dt_{max}$  (Fig. 3A) and LV  $dP/dt_{min}$  (Fig. 3B) without a change in heart rate (Fig. 3D), consistent with the known characteristics of AngII on myocardial function (21). However, no difference was observed between the TG GRK5 mice compared with nontransgenic controls in the response to administered AngII (Fig. 3), indicating that despite the extraordinarily high levels of GRK5 expressed in the hearts of these animals, no AngII receptor desensitization is apparent. LV end diastolic pressure was also not statistically different between groups at baseline (control  $1.2 \pm 0.5$  versus transgenic  $0.8 \pm 0.3$  mmHg) or with AngII (control  $4.6 \pm 0.8$  versus transgenic  $3.2 \pm 0.4$  mmHg maximal dose). Thus, these data strongly suggest that myocardial overexpression of GRK-5 results in selective uncoupling of G-protein coupled receptors *in vivo*.

As described above, we have previously reported increased desensitization of the  $\beta$ -AR in transgenic mice with cardiac overexpression of  $\beta$ ARK1 due to a decrease in functional G protein coupling of receptors (8). Since overexpression of GRK5 results in selective uncoupling of myocardial G protein-coupled receptors, we asked the question whether this was unique to GRK5, or could it be a property of other members of the family. *In vivo* physiologic measurements, in response to infusions of AngII were performed in transgenic animals that overexpress cardiac targeted  $\beta$ ARK1 (TG  $\beta$ ARK12). In contrast to the GRK5 overexpressing animals, the AngII-induced rise in LV  $dP/dt_{max}$  was significantly attenuated in the TG  $\beta$ ARK12 mice compared with nontransgenic littermate controls (Fig. 4A). The enhancement of relaxation with AngII administration was also attenuated (Fig. 4B). The increase in LV systolic pressure was similar in both groups, except for the highest dose of AngII, which was slightly lower in the TG  $\beta$ ARK12 mice (Fig. 4C). Heart rate in the TG  $\beta$ ARK12 mice was significantly lower at all time points compared with controls but was unaffected by AngII infusion (Fig. 4D). LV end diastolic pressure was not statistically different between groups at baseline (control  $4.4 \pm 0.6$  versus transgenic  $6.2 \pm 1.1$  mmHg) or with AngII (control  $8.0 \pm 0.8$  versus transgenic  $9.1 \pm 0.8$  mmHg for the maximal dose). Thus, these *in vivo* effects seen in TG  $\beta$ ARK12 animals indicate that the cardiac AngII receptor is a target for  $\beta$ ARK1-mediated desensitization and demonstrates a significant qualitative difference in the *in vivo* substrates of these two GRKs.

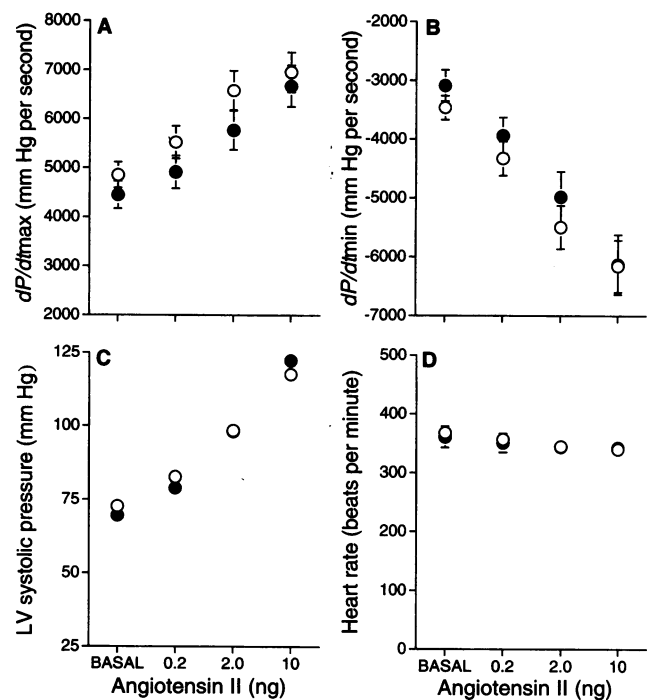


FIG. 3. *In vivo* assessment of LV function of TG GRK5-45 ( $n = 15$ ) (●) and nontransgenic control mice ( $n = 17$ ) (○) in response to progressive doses of AngII. Cardiac catheterization was performed and the following parameters are shown: (A) LV  $dP/dt_{max}$ ; (B) LV  $dP/dt_{min}$ ; (C) LV systolic pressure; and (D) heart rate. No significant difference between groups was found in response to AngII (ANOVA). In both groups, a significant difference was found for both the AngII 2-ng and 10-ng dose compared with their respective basal levels for (A)  $dP/dt_{max}$  ( $P < 0.001$ ); (B)  $dP/dt_{min}$  ( $P < 0.01$ ); and (C) LV systolic pressure ( $P < 0.01$ ).

Although LV  $dP/dt_{max}$  is a sensitive index of contractility, it is influenced by loading conditions, particularly preload (22). Since LV end diastolic pressure was not significantly different between groups for any of the above experiments, it is unlikely that the observed alterations in receptor function were due to differences in end diastolic pressure. To determine whether acute changes in systolic loading conditions can affect LV  $dP/dt_{max}$ , a pharmacological approach was used. In separate experiments in control mice ( $n = 4$ ), acute increase in LV systolic pressure was achieved with progressive doses (0.025–2.5 mg) of the nitric oxide synthase inhibitor L-NAME. As shown in Fig. 5, LV  $dP/dt_{max}$  was unchanged ( $5271 \pm 361$  to  $5116 \pm 375$  mmHg/sec) despite a 40 mmHg ( $73 \pm 5$  to  $112 \pm 8$  mmHg) increase in LV systolic pressure after L-NAME infusion. The increase in systolic pressure was similar to that observed with AngII infusion. These data suggest that systolic loading conditions alone are unlikely to cause the above findings and supports the conclusion that the AngII receptor

Table 2. Hemodynamic parameters in conscious mice following recovery from anesthesia

Parameter	Control, $n = 7$		TG GRK5-45 $n = 7$	
	Anesthetized	Conscious	Anesthetized	Conscious
LVSP, mmHg	$93 \pm 5$	$120 \pm 4^*$	$94 \pm 4$	$110 \pm 6^*$
LVEDP, mmHg	$12.5 \pm 1.5$	$5.7 \pm 1.0^*$	$9.3 \pm 1.5$	$9.2 \pm 2.0$
LV $dP/dt_{max}$ , mmHg/s	$5432 \pm 378$	$14773 \pm 1303^\dagger$	$5024 \pm 300$	$7998 \pm 971^\ddagger$
LV $dP/dt_{min}$ , mmHg/s	$-4901 \pm 183$	$-10487 \pm 1270^\dagger$	$-4866 \pm 378$	$-5922 \pm 802^\ddagger$
Heart rate, bpm	$239 \pm 13$	$481 \pm 24^\dagger$	$185 \pm 7$	$428 \pm 38^\ddagger$

LVSP, LV systolic pressure; LVEDP, LV end diastolic pressure; LV  $dP/dt_{max,min}$ , maximal and minimal first derivative of LV pressure, respectively; and bpm, beats per min.

\* $P < 0.05$ , conscious versus anesthetized for either group.

$^\dagger P < 0.001$ , conscious versus anesthetized for either group.

$^\ddagger P < 0.001$ , TG GRK5 conscious versus control conscious.

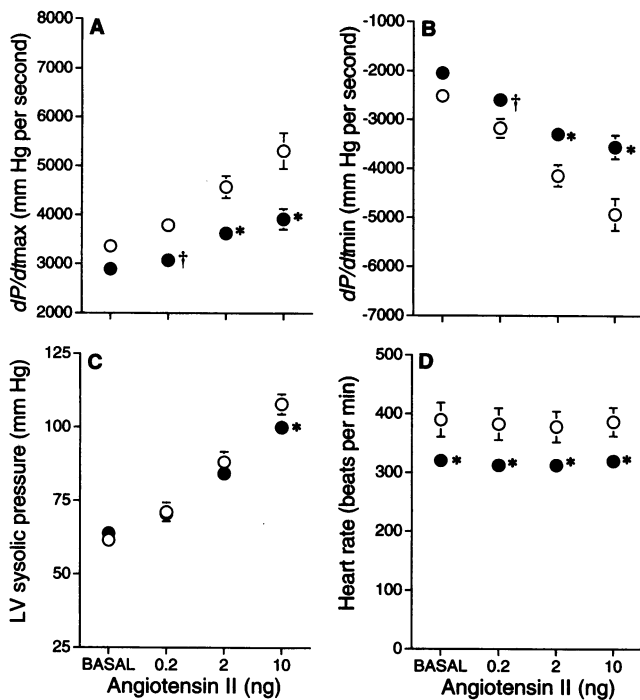


FIG. 4. Effect of AngII on *in vivo* LV function of transgenic mice overexpressing  $\beta$ ARK1. Cardiac catheterization was performed in TG  $\beta$ ARK12 ( $n = 9$ ) ( $\bullet$ ) and negative control mice ( $n = 9$ ) ( $\circ$ ). Measured parameters are shown at baseline and after progressive doses of AngII: (A) LV  $dP/dt_{max}$ ; (B) LV  $dP/dt_{min}$ ; (C) LV systolic pressure; and (D) heart rate. \*,  $P < 0.001$ ; and †,  $P < 0.05$ ; control versus transgenic, ANOVA. A significant between-group main effect in response to AngII was found for (A)  $dP/dt_{max}$  ( $P < 0.001$ ); (B)  $dP/dt_{min}$  ( $P < 0.01$ ); and (D) heart rate ( $P < 0.05$ ). The pattern of change between groups was statistically significant for (A)  $dP/dt_{max}$  ( $P < 0.05$ ); (B)  $dP/dt_{min}$  ( $P < 0.05$ ); and (C) LV systolic pressure ( $P < 0.01$ ).

is uncoupled in the TG  $\beta$ ARK12 mice while it is not modified in TG GRK5-45 mice.

## DISCUSSION

This study demonstrates that desensitization of G protein-coupled receptors *in vivo* by GRKs, such as GRK5, is not uniform. Furthermore it appears that  $\beta$ ARK1 can affect at least two divergent signaling pathways by uncoupling both the  $\beta$ -AR and AngII receptor. These results provide an example

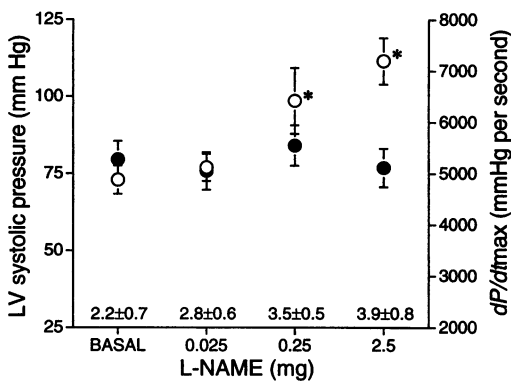


FIG. 5. Effect of the nitric oxide synthase inhibitor L-NAME on contractile function in control mice ( $n = 4$ ). Simultaneous LV systolic pressure ( $\circ$ ) and LV  $dP/dt_{max}$  ( $\bullet$ ) are plotted before and after progressive doses (0.025–2.5 mg) of L-NAME. Numbers on graph represent LV end diastolic pressure at each time point, which were not significantly different from basal. (\*,  $P < 0.001$  versus basal).

of the power of these transgenic animal models to elucidate critical issues about physiological receptor specificity of the important GRK enzymes.

The extent of diminution of the *in vivo* myocardial responses to isoproterenol in these GRK5 overexpressing animals is more pronounced than that of the responses in transgenic mice with cardiac-specific overexpression of  $\beta$ ARK1 (TG  $\beta$ ARK12) (8). This is likely attributable to the greater overexpression of GRK5 in these animals (>30-fold) versus the 3- to 5-fold increase over endogenous  $\beta$ ARK1 in the TG  $\beta$ ARK12 animals (8). Importantly, these results demonstrate that a second GRK, in addition to  $\beta$ ARK1, can affect myocardial  $\beta$ -AR signaling *in vivo* and hence regulate cardiac function.

**Effect of Anesthesia.** Baseline contractility and responsiveness to  $\beta$ -AR stimulation may be profoundly altered by general anesthesia (19, 20). Anesthesia depresses myocardial contractility and can significantly affect systolic and diastolic properties of the heart (23). Since autonomic mechanisms are important in the regulation of myocardial contractility under conscious conditions, we tested whether GRK5 overexpression would result in a cardiac phenotype similar to that found under anesthetized conditions. Our data demonstrate that in the conscious mouse, with integrated neurohumoral pathways, forced overexpression of GRK5 chronically uncouples myocardial  $\beta$ -ARs.

The choice of anesthetic agent clearly influences the effect on hemodynamic parameters. We used a combination of ketamine and xylazine, which results in marked bradycardia because of the CNS depression induced with xylazine. Indeed, the bradycardia observed with anesthesia in our mice is quite different than that reported with the use of barbiturate anesthetics, which tend to increase sympathetic activation (19, 20). The recovery from anesthesia in mice used in this study, was related to withdrawal of the central nervous system depression and accounts for the dramatic increase in heart rate and  $dP/dt_{max}$  in the awake mouse compared with that under anesthesia. Furthermore these results illustrate an important principle with respect to  $\beta$ -AR signal transduction in that receptor occupancy by agonist is a required process for homologous desensitization of the  $\beta$ -AR (6, 12). The anesthetic agents used in this study would depress sympathetic outflow and result in low levels of norepinephrine, the endogenous ligand for the  $\beta$ -AR, which may account for only the small difference in basal contractility between the TG GRK5 and control mice. In contrast, recovery from anesthesia would be associated with an increase in neuronal norepinephrine release, now interacting with uncoupled  $\beta$ -ARs, to result in the TG GRK5-45 cardiac phenotype. This process is also apparent in the isoproterenol stimulation studies (Fig. 2).

**Influence of Loading Conditions and Heart Rate.** Increases in either LV end diastolic pressure or heart rate will positively influence LV  $dP/dt_{max}$ . In our studies, although LV end diastolic pressure increased slightly with agonist infusion, no difference was observed between the transgenic and control groups, making it unlikely that the diminished LV  $dP/dt_{max}$  response was the result of changes in preload alone. The small increase in LV end diastolic pressure with isoproterenol infusion is expected, since each mouse receives  $\approx 500 \mu\text{l}$  of volume for the agonist studies and would tend to counter the expected fall in preload with  $\beta$ -AR stimulation.

Heart rate will importantly affect contractile function as shown in mice following recovery from anesthesia. Mice overexpressing  $\beta$ ARK1 had a significantly lower heart rate compared with control mice. This likely results from atrial expression of the transgene with the  $\alpha$ -myosin heavy chain promoter. It is interesting that heart rate was not different in the TG GRK5 mice using the same promoter to drive expression and may relate to the ability of  $\beta$ ARK1 to desensitize other G protein-coupled receptors *in vivo*. Although the heart rate under basal conditions is lower in the TG  $\beta$ ARK12 mice,

the response to AngII was very different for contractility, as shown by the blunted increase in  $dP/dt_{\max}$  compared with the parallel downward shift in the heart rate curve. The different pattern of change in the  $dP/dt_{\max}$  response between the groups is confirmed by the significant interaction effect in the ANOVA analysis. These data imply that despite the small difference in basal heart rate, the effect of AngII on contractility was significantly different between the transgenic and control mice consistent with chronic uncoupling of the AT<sub>1</sub> receptor.

Since AngII is known to stimulate release of catecholamines from the adrenal medulla and peripheral sympathetic nerve endings, it is possible that the increase in  $dP/dt_{\max}$  was the result of heightened  $\beta$ -AR stimulation (24). We feel this is unlikely, since our data shows the TG GRK5 mice to have a markedly desensitized  $\beta$ -AR signal pathway, which should have blunted the effect of AngII if it were mediated through the  $\beta$ -AR. Thus, the mechanism for the increase in contractile function with AngII infusion in our preparation is more likely to be mediated through the Gq-coupled AT<sub>1</sub> receptor, which activates phospholipase C to generate the second messengers, inositol trisphosphate, and diacylglycerol and stimulate intracellular Ca<sup>2+</sup> release (3, 4). Our results are also consistent with *in vitro* studies demonstrating much greater effects of  $\beta$ ARK1 than GRK5 on AngII receptor function in transfected cell systems (14).

**Implications.** These results have important implications in heart failure where elevated mRNA levels for  $\beta$ ARK1 and enhanced GRK activity (9) may act to desensitize  $\beta$ -ARs, leading to the characteristic observation of reduced  $\beta$ -AR responsiveness to catecholamines (25, 26). Our results suggest that upregulation of GRK5 in heart failure could contribute to  $\beta$ -AR uncoupling and may provide another attractive target when attempting to modulate receptor desensitization in the failing heart (27). Furthermore, this study suggests that *in vivo*, enhanced receptor phosphorylation may not be limited to the  $\beta$ -AR but it can involve other G protein-coupled receptors.

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