Myocardial overexpression of GRK3 in transgenic mice: evidence for in vivo selectivity of GRKs

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Iaccarino, Guido, Howard A. Rockman, Kyle F. Shotwell, Eric D. Tomhave, and Walter J. Koch. Myocardial overexpression of GRK3 in transgenic mice: evidence for in vivo selectivity of GRKs. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1298–H1306, 1998.—Transgenic mice were generated with cardiac-specific overexpression of the G protein-coupled receptor kinase 3 (GRK3) to explore the in vivo role of this GRK in cardiac function. GRK3 is expressed in the heart along with the β-adrenergic receptor kinase (β-ARK1) and GRK5. We have previously demonstrated that myocardial targeted overexpression in transgenic mice of β-ARK1 (Koch, W.J., H.A. Rockman, P. Samama, R.A. Hamilton, R.A. Bond, C.A. Milano, and R.J. Lefkowitz. Science 268: 1350–1353, 1995) or GRK5 (Rockman, H.A., D.-J. Choi, N.U. Rahman, S.A. Akhter, R.J. Lefkowitz, and W.J. Koch. Proc. Natl. Acad. Sci. USA 93: 9954–9959, 1996) results in significant attenuation of β-adrenergic signaling and in vivo cardiac function and selective desensitization of angiotensin (ANG) II–mediated cardiac responses. Surprisingly, myocardial overexpression of GRK3 resulted in normal biochemical signaling through β-adrenergic receptors (β-ARs), and in vivo hemodynamic function in response to a β-AR agonist was indistinguishable from that in nontransgenic controls. Furthermore, in vivo signaling and functional responses to ANG II were unaltered. However, myocardial thrombin signaling, assessed by p42/p44 mitogen-activated protein (MAP) kinase activation, was significantly attenuated in GRK3 transgenic mouse hearts, indicating a distinct in vivo substrate specificity for GRK3.

β-adrenergic receptor; thrombin receptor; G protein signaling; desensitization; cardiac contractility

AS A FAMILY OF serine/threonine kinases, the G protein-coupled receptor kinases (GRKs) phosphorylate G protein-coupled receptors, resulting in functional uncoupling, a process known as desensitization (15). There are six known GRK family members that share several structural and mechanistic features, including recognizing only the agonist-occupied form of receptors (8). GRKs are in general ubiquitously expressed, and multiple kinases are present in several tissues. In the heart, several G protein-coupled receptors play important roles in regulating cardiac function (22). For example, β-adrenergic receptors (β-ARs) are responsible for increasing the rate and force of myocardial contraction in response to stress and exercise (22).

Thus regulation of these myocardial G protein signaling pathways is critical to normal cardiac physiology. The major GRKs expressed in the heart are the β-adrenergic receptor kinase (β-ARK1), GRK3 (β-ARK2), and GRK5 (8). An increasing body of evidence is emerging indicating that GRKs are critical regulators of myocardial adrenergic signaling and cardiac function. For example, myocardial β-ARK1 (22) expression and activity has been shown to be increased in several cardiovascular diseases in which β-AR responsiveness is attenuated, including severe human heart failure (27), myocardial ischemia (28), and pressure-overload ventricular hypertrophy (3). To date, little is known about other myocardial GRKs in pathological conditions because most studies have targeted β-ARK1.

GRK3 is considered to be an isozyme of β-ARK1 sharing an overall sequence identity of 85% (2). GRK3 is found in nearly all the same tissues as β-ARK1, although to a much lower extent (2); thus the reason for the existence of these isoforms, especially in the heart, remains unclear. It has been shown that β-ARK1, GRK3, and GRK5 share several in vitro G protein-coupled receptor substrates (7, 8, 16); however, in vivo analysis has been limiting and difficult to dissect. Exceptions appear to be the olfactory receptor system, in which GRK3 has been clearly demonstrated to be the relevant GRK (19, 23). Furthermore, in vitro evidence exists for selectivity of GRK3 versus β-ARK1 for the thrombin receptor (9). Previously, we have used transgenic technology to overexpress β-ARK1 (14) or GRK5 (21) specifically in the hearts of mice and have demonstrated profound effects on β-AR signaling and cardiac physiology. In addition, physiological studies with angiotensin (ANG) II demonstrated that GRKs do selectively distinguish in vivo receptor substrates (21). The present study was carried out to investigate the role of GRK3 in myocardial adrenergic regulation and to explore in vivo substrate specificity of this GRK. GRK3 was targeted exclusively to the heart with the use of the murine α-myosin heavy chain (α-MHC) gene promoter, and β-adrenergic signaling in vitro and in vivo were investigated. In addition, in vivo cardiac responses to ANG II and thrombin were studied. Results presented reveal that GRK3 has unique in vivo substrate selectivity.

METHODS

Generation and identification of transgenic mice. Transgenic mice were created with the use of the α-MHC promoter (24) to direct cardiac-specific overexpression of GRK3 (β-ARK2). The GRK3 transgene was constructed by inserting the −2.1-kb cDNA encoding the entire open reading frame for
bovine GRK3 (2) into a previously constructed transgene plasmid that contains the 5.5-kb α-MHC promoter and the simian virus 40 (SV40) intron poly(A) signal (14, 21) to yield pGEM-α-MHC-GRK3-SV40 (Fig. 1A). This construct was then linearized and purified before pronuclear injections were done by the Duke Comprehensive Cancer Center Transgenic Facility. The mouse strain used was C57/B16 as in our previous studies (14, 21, 22). Originally, five founder lines were established (TG GRK3–3, -27, -42, -112, and -114). Total heart RNA isolated from F1 pups of these five lines were analyzed for transgene message, and two lines, TG GRK3–27 and TG GRK3–114, had significant and similar mRNA expression (data not shown). Litter sizes and postneonatal development of these transgenic animals were indistinguishable from those of nontransgenic littermate controls (NLCs). Offspring were screened by Southern blot analysis with a probe for the SV40 sequence (14, 21). Second-generation animals 2–5 mo of age were used for all studies.

Heart-to-body weight ratio. Mice were weighed, deeply anesthetized with a mixture of ketamine and xylazine as described previously (14, 21), and their hearts quickly excised. The hearts were rinsed quickly in ice-cold PBS, blotted dry on absorbent paper, and weighed. Heart-to-body weight ratios are given in milligrams per gram. After hearts were weighed, they were snap frozen in liquid N2 and stored at −80°C until used for biochemical assessment.

Protein immunoblotting. Immunodetection of myocardial levels of GRK3 was performed on detergent-solubilized extracts after immunoprecipitation. Excised hearts were homogenized in ice-cold radioligand immunoprecipitation assay (RIPA) buffer [50 mM Tris·HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 5 mM EGTA, 10 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. GRK3 was immunoprecipitated from 1 ml of clarified extract (equal protein amounts) with a 1:2,000 (0.5 µl) monoclonal anti-β-ARK1/2 (GRK2/3) antibody (3, 16) and 35 µl of a 50% slurry of protein A-agarose conjugate agitated for 1 h at 4°C as described previously (3). Immune complexes were then washed three times in ice-cold RIPA, and the washed agarose beads were resuspended in 35 µl of protein gel loading buffer, heated at 85°C for 5 min, and then electrophoresed through 12% polyacrylamide Tris-glycine gels and transferred to nitrocellulose. The ~80-kDa GRK3 protein was visualized with either the monoclonal antibody used for immunoprecipitation or a commercial polyclonal antibody selective for GRK3 (Santa Cruz Biotechnology) and enhanced chemiluminescence (ECL, Amersham) detection of anti-mouse IgG horseradish peroxidase (3). Quantitation was done by scanning the final autoradiography films and using ImageQuant software (Molecular Dynamics).

GRK activity by rhodopsin phosphorylation. Myocardial extracts were prepared by homogenization of excised hearts in 2 ml of ice-cold lysis buffer [25 mM Tris·HCl (pH 7.5), 5 mM EDTA, 5 mM EGTA, 10 µg/ml leupeptin, 20 µg/ml aprotinin, and 1 mM PMSF] as described previously (3, 14, 21). Soluble cytosolic fractions and membrane fractions were separated, and GRK activity was assessed by light-dependent phosphorylation of rhodopsin and gel electrophoresis as previously described (3, 14, 21). Some experiments were done with the addition of purified G protein βγ-subunits (Gβγ) for maximal activation of GRK3 (8). Phosphorylated rhodopsin was visualized by autoradiography of dried gels and quantified using a phosphorimager (Molecular Dynamics).

β-AR radioligand binding. Myocardial membranes were prepared by homogenization of excised hearts in ice-cold binding buffer [50 mM HEPES (pH 7.3), 150 mM KCl, and 5 mM EDTA] as we have described previously (14, 21). Receptor binding was performed as previously described (14, 21) using the 125I-labeled β-AR ligand cyanopindolol. Nonspecific binding was determined in the presence of 20 µM alprenolol. Reactions were conducted in 500 µl of binding buffer at 37°C for 1 h and then terminated by suction through glass-fiber filters. All assays were performed in triplicate, and receptor number (in fmol) was normalized to milligrams of membrane protein.

**Fig. 1.** Transgenic construct and assessment of myocardial G protein-coupled receptor kinase 3 (GRK3) overexpression. A: DNA construct used for generation of GRK3-overexpressing transgenic mice. β-ARK2, β-adrenergic receptor kinase 2; SV40, simian virus 40. B: Immunodetection of myocardial levels of GRK3 using a selective antibody from nontransgenic littermate control (NLC) and TG GRK3–27 mice (a line of transgenic GRK3 mice). An aliquot of purified GRK3 was used to determine the accurate size of the transgene product. Histogram shows means ± SE in densitometer units of scanned blots for n = 4–5 hearts each. *P < 0.05 vs. NLC (Student’s t-test). C: Representative immunoblots with a nonselective monoclonal antibody (3, 17) showing GRK2/3 overexpression in cytosolic and membrane fractions from hearts of TG GRK3–27 mice and transgenic mice overexpressing β-ARK1 (GRK2) (TG β-ARK12 mice) (14). Molecular mass is shown in kDa.
Adenyl cyclase activity. Crude myocardial membranes were prepared as described previously (14, 21) from both transgenic and nontransgenic hearts. Membranes (20–30 µg of protein) were incubated for 15 min at 37°C with [α-32P]ATP under basal conditions or in the presence of either 100 µM isoproterenol or 10 mM NaF, and cAMP was quantitated by standard methods as we have previously described (14, 21). To evaluate acute desensitization, NLCs and TG GRK3 mice were injected (intraperitoneally) with either saline or isoproterenol (150 mg/kg) and killed 30 min later. Myocardial membranes were prepared, and adenyl cyclase assays were repeated as described above. Desensitization, measured as the percent decrease in maximal isoproterenol stimulation-induced isoproterenol pretreatment, was determined as we have described previously (6).

Physiological evaluation. In vivo hemodynamic measurements in anesthetized mice were done essentially as described previously (3, 14, 21). Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg). After endotracheal intubation, mice were connected to a rodent ventilator. After bilateral vagotomy, the chest was opened and a 1.8-Fr 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 s after injection of incremental doses of isoproterenol or ANG II. Continuous high-fidelity LV pressure was recorded at baseline and 45–60 s after each dose of agonist on an eight-channel chart recorder and in digitized form at 2,000 Hz for later analysis. Experiments were then terminated with an overdose of pentobarbital sodium. Hearts were rapidly excised, and individual chambers were separated, weighed, and then frozen in liquid N2 for later analysis. Parameters measured were heart rate (HR), LV systolic and end-diastolic pressure, and the maximal and minimal first derivative of LV pressure (LV dP/dt max and LV dP/dt min, respectively). Ten sequential beats were averaged for each measurement.

Mitogen-activated protein kinase activity. Myocardial mitogen-activated protein (MAP) kinase activation was determined from ventricular injections as we have described previously (14). Briefly, mice were anesthetized, the heart was isolated through a left thoracotomy, and a direct LV intracavitary injection of 50 µl of PBS, 100 mM ANG II, or SL95RR (thrombin receptor agonist peptide, 100 or 500 µM) was administered. Ninety seconds after injection, the heart was excised while still beating and was snap frozen in liquid N2. The hearts were homogenized in 2 ml of RIPA buffer, and MAP kinases were immunoprecipitated for 2 h at 4°C with anti-extracellular signal-regulated kinase 2 antibody (Santa Cruz Biotechnology) in protein A-agarose as described previously (1). Immune complexes were washed twice with RIPA buffer [20 mM HEPES (pH 7.0), 10 mM MgCl2, and 1 mM dithiothreitol], and final pellets were resuspended in 40 µl of reaction buffer [kinase buffer with 0.25 mg/ml myelin basic protein (MBP), 20 µM ATP, and 20 µCi/ml [γ-32P]ATP] and incubated at room temperature for 30 min. The reactions were quenched with 2X Laemmli buffer and electrophoresed through a 4–20% Tris-glycine gradient gel (Novex), and phosphorylated MBP was visualized by autoradiography (1). The extent of MBP phosphorylation was determined using the PhosphorImager.

Statistical analysis. Data are expressed as means ± SE. Unpaired Student’s t-tests were performed as appropriate. Analysis of variance for repeated measurements with a grouping factor was performed to evaluate changes in the hemodynamic parameters among the NLCs and the TG GRK3 groups. Newman-Keuls test was conducted as post hoc analysis with regard to differences in mean values among the groups at a specific dose of infused agonist.

RESULTS

GRK3 overexpression in hearts of transgenic mice. Two independent lines were identified by Northern analysis of total heart RNA as having high levels of GRK3 transgene mRNA expression (data not shown). These two lines, designated as TG GRK3–27 and TG GRK3–114, were used throughout this study and compared with nontransgenic litter mates (NLCs) used as controls. Heart sizes from TG GRK3–27 mice were similar to those of NLCs as determined by heart-to-body weight ratio [TG GRK3–27: 4.76 ± 0.11 vs. NLC: 4.81 ± 0.21 mg/g, P = not significant (NS)].

Myocardial GRK3 content was identified in NLC and TG GRK3 animals via solubilization of hearts and immunoprecipitation, followed by visualization with Western blotting using an antibody selective for GRK3 (Fig. 1B). Quantification of immunoreactive GRK3 in the hearts of TG GRK3–27 and TG GRK3–114 animals revealed similar expression levels, with TG GRK3–27 animals having slightly higher GRK3 overexpression that was ~12-fold greater than endogenous levels of this GRK (Fig. 1B). Using a monodonal antibody that does not distinguish between GRK3 and βARK1 (GRK2) (16), we compared GRK2/3 overexpression in TG GRK3–27 animals with our previously generated overexpression of βARK1 (TG βARK12) in transgenic mice (14). This analysis revealed that TG GRK3 animals had approximately twice as much GRK overexpression (Fig. 1C). GRK3 overexpression in TG GRK3–27 hearts, like β-ARK1 overexpression in TG βARK12 hearts, was found in both soluble and membrane fractions (Fig. 1C).

To further quantify GRK3 overexpression, cardiac extracts were assayed for in vitro phosphorylation activity toward the G protein-coupled receptor substrate rhodopsin. Consistent with GRK3 protein overexpression, there was a marked increase in kinase activity in TG GRK3 cytosolic myocardial extracts (Fig. 2A). The cytosol is the main cellular compartment for GRK2 and 3 (8), and soluble cardiac GRK activity was approximately eightfold greater for TG GRK3–27 animals than for NLCs. Membrane extracts from TG GRK3–27 hearts also possessed higher GRK activity (data not shown). As with the protein results (Fig. 1C), kinase activity in TG GRK3 extracts was greater than in extracts from TG βARK12 animals overexpressing GRK2, demonstrating that total myocardial GRK activity is significantly greater in TG GRK3–27 mice (data not shown). In vivo activation of GRK3, as with GRK2 (β-ARK1), involves membrane translocation that is directed by the disassociated βγ-subunits of activated G proteins (Gβγ) (8, 15). This is mediated by the direct binding of Gβγ to a specific region within the carboxyl terminus of GRK3 (13, 20). To examine this interaction in animals overexpressing GRK3, we carried out rhodop- sin phosphorylation assays of myocardial extracts with the addition of purified Gβγ. Myocardial GRK activity
CARDIAC GRK3 OVEREXPRESSION IN TRANSGENIC MICE

Assessment of myocardial GRK activity in transgenic mice. A: in vitro rhodopsin (Rho) phosphorylation assay using myocardial extracts from NLC and GRK3 transgenic mice. Histogram shows means ± SE of phosphate incorporation for n = 4-5 hearts each. *P < 0.05 vs. NLC (Student’s t-test). TG GRK3–27, a line of transgenic GRK3 mice. Inset: representative autoradiograph of a dried gel on which phosphorylated Rho is visualized. B: representative autoradiograph of phosphorylated Rho comparing myocardial GRK activity in TG GRK3–27 and NLC hearts without (−) and with (+) purified Gβγ.

In TG GRK3 extracts was doubled by αβγ addition, demonstrating appropriate GRK3 activation (Fig. 2B).

Assessment of myocardial β-adrenergic signaling in TG GRK3 mice. To examine the biochemical effects of GRK3 overexpression on the myocardial β-AR system, β-AR density was examined in cardiac membranes along with coupling to the effector enzyme adenylyl cyclase. There was no difference in β-AR density between TG GRK3–27 (42 ± 11 fmol/mg membrane protein, n = 4) and NLC hearts (46 ± 3 fmol/mg membrane protein, n = 4, P = NS). Table 1 summarizes the myocardial adenylyl cyclase activities in both lines of TG GRK3 mice compared with activities in NLCs. Basal adenylyl cyclase activity as well as stimulation by the β-agonist isoproterenol was not altered in membranes overexpressing GRK3. To further evaluate β-AR signaling in TG GRK3 mice, we investigated the acute desensitization of receptors by injecting NLC and TG GRK3 mice with either saline or isoproterenol 30 min before death and preparation of myocardial membranes (n = 4 in each condition). Adenylyl cyclase assays were repeated with isoproterenol stimulation, and our results showed that the β-ARs in both NLC and TG GRK3 hearts desensitized to the same level (NLC: 29 ± 11% vs. TG GRK3: 32 ± 8%, P = NS), demonstrating that the overexpression of GRK3 did not enhance β-AR uncoupling. The lack of β-AR desensitization in TG GRK3 animals is in marked contrast to what was found in cardiac membranes isolated from transgenic hearts overexpressing βARK1 (14) or GRK5 (21), where there was significant blunting of the myocardial adenylyl cyclase system.

In vivo assessment of cardiac function in TG GRK3 mice. To directly assess whether overexpression of GRK3 affects the β-AR-mediated myocardial physiology of these mice, cardiac catheterization was used to measure catecholamine responsiveness in vivo in the intact anesthetized mouse (Fig. 3). LV hemodynamics were measured continuously before and after progressive doses of isoproterenol were injected. Parameters studied in addition to HR and shown in Fig. 3 were LV systolic pressure, LV end-diastolic pressure, and LV dP/dtmax and LV dP/dtmin as measures of cardiac contractility and relaxation, respectively. Consistent with the biochemical data, basal cardiac function was not different in TG GRK3–27 animals compared with NLCs (Fig. 3, A–D). In addition, responses to isoproterenol were not altered by GRK3 overexpression. Baseline HR was similar between groups (NLC: 468 ± 12 vs. TG GRK3: 450 ± 9 beats/min, P = NS) and showed the same response to isoproterenol (NLC: 551 ± 12 vs. TG GRK3: 538 ± 120 beats/min for 1,000 pg isoproterenol, P = NS). Similar hemodynamic results were obtained in TG GRK3–114 animals (data not shown). Surprisingly, these data indicate that, in vivo, myocardial β-ARs (more specifically, β1-ARs) are not targets for GRK3-mediated desensitization, which differs significantly from what has been demonstrated in vitro (7). Furthermore, these results are in contrast to what has been observed in transgenic mice with cardiac overexpression of βARK1 or GRK5, where in vivo β-AR responses were severely attenuated (14, 21).

We also assessed in vivo cardiac physiological responses to the G protein-coupled receptor agonist ANG II to determine whether this critical receptor system is a target for GRK3 action in the heart (Fig. 4). We have previously shown that ANG II can lead to increased myocardial contractility in the anesthetized mouse.

Table 1. Adenylyl cyclase activity in myocardial membranes isolated from nontransgenic littermate control and transgenic GRK3 hearts

<table>
<thead>
<tr>
<th>Hearts</th>
<th>Basal</th>
<th>Iso</th>
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<tr>
<td>NLC</td>
<td>16.4 ± 1.1</td>
<td>22.9 ± 1.1*</td>
<td>351 ± 31</td>
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<tr>
<td>TG GRK3–27</td>
<td>14.6 ± 1.0</td>
<td>21.0 ± 1.1*</td>
<td>350 ± 20</td>
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<tr>
<td>TG GRK3–114</td>
<td>16.3 ± 0.6</td>
<td>24.1 ± 0.6*</td>
<td>355 ± 65</td>
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Data are means ± SE expressed as % activity found with 10 mM NaF (expressed as pmol·mg⁻¹·min⁻¹) for basal and isoproterenol (Iso, 10⁻⁴ M)-stimulated activity; n = 3–6 hearts for each group done in triplicate. NLC, nontransgenic littermate control; TG GRK3–27 and -114, transgenic G protein-coupled receptor kinase 3 mice, founder lines 27 and 114, respectively. *P < 0.02, Iso response vs. basal values (Student’s t-test).
ANG II is a potent vasoconstrictor as demonstrated by the rise of LV systolic pressure (Fig. 4C). Likewise, LV contractility increased in response to ANG II (Fig. 4A). Cardiac responses to ANG II in TG GRK3–27 mice were not different compared with responses in control animals (Fig. 4, A–D), demonstrating that, like β-ARs, ANG II receptors are not targets for GRK3 in the heart. This again is in contrast to previous in vitro studies showing that GRK3 could phosphorylate and desensitize ANG II receptors (17). Although basal HR was slightly lower in the mice with GRK3 overexpression (NLC: 485 ± 610 vs. TG GRK3: 440 ± 13 beats/min, \( P < 0.05 \)), the HR response to ANG II infusion was similar for both groups. The reason for this small but statistically significant difference in basal HR between NLC and TG GRK3 mice is likely due to physiological variability of the in vivo experiments. Importantly, however, the response to ANG II was the same for both groups, suggesting that in vivo ANG II receptors are not targets for GRK3. These results for GRK3 are similar to what was observed in transgenic mice with cardiac-specific overexpression of GRK5; however, β-ARK1 overexpression in the hearts of transgenic mice can lead to desensitization of myocardial ANG II receptors in vivo (21).

Assessment of myocardial thrombin signaling in TG GRK3 mice. Given that β-ARs or ANG II receptors are not targets for GRK3 action in vivo, other G protein-coupled receptors in the heart are apparently regulated by this kinase. One relevant signaling system to the heart that has previously been demonstrated to be regulated by GRK3 is that stimulated by the actions of thrombin (9). In cultured neonatal rat ventricular myocytes, thrombin receptor activation leads to phosphoinositide hydrolysis and activation of MAP kinase (10). Thus we investigated myocardial MAP kinase activation in NLC and TG GRK3 mice in response to the thrombin receptor-derived agonist peptide SFLLRN. In addition, we studied MAP kinase activation in response to ANG II because we have previously seen a robust signal in NLC mice (1). For examination of myocardial MAP kinase activity, anesthetized TG GRK3 and NLC mice underwent a left thoracotomy and 100 µl of the appropriate agonist was injected into the LV chamber. Hearts were excised after 90 s, and agonist-mediated LV MAP kinase activity was compared with that in hearts injected with saline. Basal MAP kinase activity measured after saline injection was not significantly different between NLC and TG GRK3 mice (data not shown). In NLC hearts, 100 µM of the SFLLRN peptide provoked a 175 ± 11% increase in MAP kinase activity, which was significantly blunted in the transgenic mouse hearts overexpressing GRK3 (Fig. 5B). In contrast, the robust activation of cardiac MAP kinase activity by ANG II injection (100 µM) was not altered by

![Fig. 3. In vivo cardiac contractility responses to isoproterenol. Hemodynamic measurements were performed in open-chest anesthetized animals (○, NLC, n = 11; ●, TG GRK3–27, n = 12), and responses were recorded at baseline and after incremental doses of isoproterenol. A: maximal first derivative of left ventricular (LV) pressure (LV dP/dt_{max}). B: minimal first derivative of LV pressure (LV dP/dt_{min}). C: LV systolic pressure. D: LV end-diastolic pressure. No significant difference among groups was found for measured parameters.](image-url)
GRK3 overexpression (Fig. 5), which is consistent with our observed physiological data (Fig. 4). These data indicate that myocardial thrombin receptors are in vivo targets for GRK3-mediated desensitization and demonstrate that the GRK3 overexpressed in the hearts of these transgenic animals is functionally capable of regulating certain G protein-coupled receptor signaling; however, β-AR regulation is surprisingly not an in vivo target for GRK3 regulation, even when the kinase is considerably overexpressed.

**DISCUSSION**

This study was designed to examine the in vivo role of GRK3 (β-ARK2) in cardiac signal transduction and function, including its role in regulating the β-AR. GRK3 is one of four GRKs found in the heart, and its in vivo receptor targets are unknown. Outside of the heart, little is known concerning the physiological role of GRK3, although it appears that GRK3 is the relevant GRK of the olfactory system (19, 23). In addition, in vitro studies in which an oocyte expression system was used have demonstrated that GRK3 can selectively desensitize signaling through thrombin receptors (9). These types of studies utilizing heterologous expression systems or purified proteins can effectively be used to characterize the enzymatic activity and molecular regulation of the various GRKs; however, in vivo substrate targets and GRK specificity are critical questions that cannot be addressed by these means. This study...
utilizes transgenic mice to elucidate physiologically relevant receptor targets of GRK3. When GRK3 is targeted to the mouse heart via the α-MHC promoter, cardiac signaling and function remains normal in response to the β-agonist isoproterenol or ANG II, whereas signaling through myocardial thrombin receptors is significantly impaired. Thus it is apparent that GRK3 has selective in vivo receptor targets.

The results concerning β-AR and ANG II signaling obtained with in vivo GRK3 overexpression are in striking contrast to our previous findings in transgenic mice overexpressing β-ARK1 (GRK2) in the myocardium, because these animals displayed significant biochemical and physiological desensitization in response to both β-agonist and ANG II infusion (14, 21). Our current findings are especially surprising because in vitro studies have demonstrated that β-ARK1 and GRK3 can phosphorylate and desensitize both β1-ARs (the predominate β-AR subtype in myocardium) and ANG II receptors (7, 17). Furthermore, these results are especially significant because the level of GRK3 overexpression in the hearts of these transgenic mice is greater than the level of β-ARK1 overexpression in TG β-ARK12 mice (Fig. 1). Thus it is clear that these two GRKs play distinct in vivo roles in the normal regulation of myocardial function.

Our current findings are indeed surprising given that β-ARK1 and GRK3 are considered isozymes because they share an overall ~85% amino acid identity approaching 95% in the catalytic domain (2). This strikingly high identity, coupled with the fact that the tissue distribution of these two GRKs is mostly overlapping (2), has led to the question of why there is this apparent redundancy in cellular G protein-coupled desensitization mechanisms. However, studies such as those described here using transgenic animal models can be extremely useful in delineating in vivo GRK selectivity.

The molecular regulation of β-ARK1 and GRK3 is a key point of emphasis when examining possible selective roles for these GRKs. Before their actions on agonist-occupied receptors, both β-ARK1 and GRK3 require a membrane-targeting event from the cytosolic component that is accomplished by specific interactions with released Gβγ and phospholipids present in the cell membrane (13, 20, 25). In vitro phosphorylation assays using myocardial extracts from TG GRK3 animals demonstrated Gβγ activation (Fig. 2B); thus the GRK3 expressed in the hearts of these mice appears to behave appropriately. The physical interaction between GRK2/3 and Gβγ takes place within the carboxyl-terminal region of these two GRKs (13, 20, 25). Peptides from this region ranging from 28 to 200 amino acids are effective in vitro inhibitors of not only GRK2/3 translocation but also other Gβγ-mediated signaling events (4, 11–13, 25). Interestingly, the most divergent region between β-ARK1 and GRK3 is located within the Gβγ-binding domain, with the highest variance found (~50% identity) within a 28-mer peptide region that has been shown to contain critical regions for the protein-protein interaction (4, 13, 25). This concentrated area of sequence diversity may reflect differential affinities of these domains from β-ARK1 and GRK3 for distinct Gβγ subunits. Thus one possible hypothesis to explain the observed in vivo GRK specificity is that Gβγ proteins released after cardiac β-AR and ANG II receptor activation have higher affinity for the carboxyl terminus of β-ARK1, whereas Gβγ proteins released after thrombin receptor activation can appropriately direct the membrane translocation of GRK3. Further studies will be required to effectively test this hypothesis; however, there is in vitro evidence demonstrating that β-ARK1 and GRK3 can be targeted to membranes in a receptor- and Gβγ-specific manner (5). This study included the finding that GRK3 but not β-ARK1 was targeted to the membrane of COS-7 cells after stimulation of endogenous thrombin receptors (5).

The fact that GRK3 can effectively desensitize thrombin receptor signaling in vivo correlates extremely well with in vitro studies previously demonstrating that GRK3 can selectively desensitize overexpressed thrombin receptors (9). Importantly, the results with thrombin signaling demonstrate that the GRK3 overexpressed in the hearts of these transgenic mice is physiologically active, supporting our conclusion that GRK3 does not regulate β-adrenergic signaling in vivo in the heart, which is the case for β-ARK1. These data suggest that β-ARK1 is the primary GRK in the heart regulating the major G protein-coupled receptor systems, whereas GRK3, expressed to a lesser degree in the heart (2), regulates other signaling pathways such as protein kinase C and MAP kinase (10). MAP kinase activation is also a major signaling component of thrombin receptors in other cell types such as smooth muscle cells (26). In this study, we found that MAP kinase activation after myocardial thrombin receptor stimulation was significantly blunted in TG GRK3 mice consistent with desensitization (Fig. 5). In contrast, MAP kinase activation after a myocardial ANG II injection was normal in TG GRK3 mice, which was consistent with our physiological findings in which myocardial contractility and pressure in response to ANG II were not altered in GRK3-overexpressing mice (Fig. 4). These results are interesting because, like ANG II receptors, thrombin receptors can couple to the G protein Gq (29). A possible explanation for these results is that these two Gq-coupled receptors activate different pools of G proteins in vivo that release distinct Gβγ subunits that can distinguish between β-ARK1 (ANG II) and GRK3 (thrombin).

Other factors in addition to Gβγ may also be involved in the in vivo GRK substrate selectivity observed in the hearts of our transgenic mice. This is evidenced by our previous findings in transgenic mice with cardiac-specific overexpression of GRK5. In these mice, it was found that myocardial β-ARs are targets of GRK5, whereas ANG II receptors are not (21). GRK5 is a kinase that is constitutively membrane bound and does not require Gβγ for its membrane targeting or activa-
tion (8, 21). Thus in vivo GRK substrate specificity involves more than receptor-specific membrane localization of the desensitizing GRK. In addition to the membrane-targeting component of GRK activation, there is evidence that GRKs contain sequences that recognize and bind to receptors. The regions of GRKs thought to be involved in receptor recognition reside in the amino terminus (8, 18), which is somewhat divergent between β-ARK1 and GRK3. Thus this is an additional area besides the Gbg-binding domain, which may explain the lack of myocardial β-AR and ANG II receptor desensitization in GRK3-overexpressing transgenic mice compared with β-ARK1 and GRK5 transgenic animals. Thus, in the milieu of the myocyte plasma membrane, the environment of these receptors may not offer a high-affinity site for GRK3 binding even when this kinase is localized to the membrane.

Taken together, our current and previous results demonstrate the usefulness of transgenic mice to explore the in vivo substrate selectivity of GRKs. Our results indicate that β-ARK1 and GRK5 may play a major role in the regulation of myocardial β-AR signaling and function, whereas GRK3 selectively regulates myocardial thronbin signaling without affecting the β-AR system. Thus these transgenic mice overexpressing three different GRKs can now be used to explore selectivity on other G-protein-coupled receptor substrates to develop a more in-depth understanding of the role of GRK-mediated desensitization in the heart.

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