Control of Myocardial Contractile Function by the Level of β-Adrenergic Receptor Kinase 1 in Gene-targeted Mice*

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We studied the effect of alterations in the level of myocardial β-adrenergic receptor kinase (βARK1) in two types of genetically altered mice. The first group is heterozygous for βARK1 gene ablation, βARK1(+/−), and the second is not only heterozygous for βARK1 gene ablation but is also transgenic for cardiac-specific over-expression of a βARK1 COOH-terminal inhibitor peptide, βARK1(+/−)/βARKct. In contrast to the embryonic lethal phenotype of the homozygous βARK1 knockout (Jaber, M., Koch, W. J., Rockman, H. A., Smith, B., Bond, R. A., Sulik, K., Ross, J., Jr., Lefkowitz, R. J., Caron, M. G., and Girod, B. 1996 Proc. Natl. Acad. Sci. U. S. A. 93, 12974–12979), βARK1(+/−) mice develop normally. Cardiac catheterization was performed in mice and showed a stepwise increase in contractile function in the βARK1(+/−) and βARK1(+/−)/βARKct mice with the greatest level observed in the βARK1(+/−)/βARKct animals. Contractile parameters were measured in adult myocytes isolated from both groups of gene-targeted animals. A significantly greater increase in percent cell shortening and rate of cell shortening following isoproterenol stimulation was observed in the βARK1(+/−) and βARK1(+/−)/βARKct myocytes compared with wild-type cells, indicating a progressive increase in intrinsic contractility. These data demonstrate that contractile function can be modulated by the level of βARK1 activity. This has important implications in disease states such as heart failure (in which βARK1 activity is increased) and suggests that βARK1 should be considered as a therapeutic target in this situation. Even partial inhibition of βARK1 activity enhances β-adrenergic receptor signaling leading to improved functional catecholamine responsiveness.

One of the most important mechanisms for rapidly regulating β-adrenergic receptor (βAR) function is agonist-stimulated receptor phosphorylation by G protein-coupled receptor kinases (GRKs) resulting in decreased sensitivity to further catecholamine stimulation (1). GRKs phosphorylate only agonist-occupied receptors leading to homologous desensitization (1, 2). The β-adrenergic receptor kinase (βARK1) is a member of a family of at least 6 GRKs, which phosphorylate and regulate a wide variety of receptors that couple to heterotrimeric G proteins (3, 4). When βARs or other G protein-coupled receptors are activated by agonist, heterotrimeric G proteins dissociate into Gα and Gβγ subunits, and the Gβγ subunit complex, which is membrane anchored by a lipid group (geranylgeranyl), can target βARK1 to the membrane through a direct physical interaction that facilitates phosphorylation of activated receptors (5, 6).

Using a transgenic based strategy for cardiac-specific over-expression of either βARK1 or a peptide inhibitor of βARK1 (βARKct), we have recently shown that in vivo, myocardial β₁-adrenergic and angiotensin II receptors are targets for βARK1 mediated desensitization (7, 8). The βARK1 inhibitor utilized is a peptide containing the carboxyl-terminal 194 amino acids of βARK1, which competes with endogenous βARK1 for Gβγ binding (7). Evidence suggesting a fundamental role for βARK1 in cardiac development was provided by gene-targeted mice in which the βARK1 gene was ablated by homologous recombination (9). Knockout mice, homozygous for the βARK1 deletion, died during mid-gestation with no viable βARK1(−/−) embryos observed past E15.5 (9). Histologic analysis revealed hypoplasia of ventricular myocardium with disorganized trabeculation. Furthermore, in vivo embryonic cardiac function demonstrated significantly impaired left ventricular (LV) ejection fraction compared with wild-type hearts, showing that βARK1 is required for normal cardiac development (9). In contrast to the complete knockout, βARK1(+/−) heterozygous animals have no obvious developmental abnormalities despite an approximate 50% reduction in the level of βARK1 protein and GRK activity (9).

In a variety of human and experimental conditions, prominent βAR desensitization in response to catecholamine stimulation has recently been shown to be associated with heightened levels of βARK1 (10–13). In chronic human heart failure, reduced agonist-stimulated adenyl cyclase activity due to both diminished receptor number and impaired receptor function is a predominant feature (14). In end-stage human heart failure, these changes in βAR function were shown to be associated with elevated mRNA levels and activity for βARK1 (10, 15). Results from transgenic mice that overexpress βARK1 and GRK5 (7, 8) demonstrate how the up-regulation of these molecules in heart failure could markedly alter βAR function by enhancing receptor desensitization. Furthermore, chronic
treatment with either the βAR antagonist bisoprolol in the pig (16) or carvedilol in the mouse (20) (a potent therapeutic agent in human heart failure, see Ref. 18), substantially decreased the level of βARK1 activity. The most compelling evidence demonstrating the importance of βARK1 in heart failure comes from a recent study whereby transgenic mice with cardiac-restricted overexpression of the βARKct were mated into a genetic model of murine heart failure achieved through ablation of the MLP gene (19). Overexpression of the βARK1 inhibitor reversed the heightened βAR desensitization in the MLP knockout mice and completely normalized cardiac function. These data strongly implicate abnormal βAR-G protein coupling in the pathogenesis of the failing heart (19). Taken together, these studies indicate the potential for a therapeutic strategy that aims to modulate the activity level of myocardial βARK1 in disease states. Decreasing the level of myocardial βARK1 in established heart failure is a novel approach to improving impaired βAR receptor function and potentially alter the pathogenesis in this disease.

In the present study, we sought to test the hypothesis that the level of cardiac βARK1 activity regulates myocardial contractile function in vivo. To test these hypotheses, we used a strategy that utilized mouse genetics to create varying levels of βARK1 activity in the heart, coupled with a physiological assessment of contractile function in the absence and presence of catecholamine stimulation.

MATERIALS AND METHODS

Experimental Animals—The gene-targeted mice used for this study were 1) heterozygous for targeted disruption of the βARK1 gene (9), and 2) offspring generated by cross breeding transgenic mice with cardiac-specific overexpression of a βARK1 inhibitor (βARKct, shown previously to have enhanced basal contractility (7)) with the βARK1(+/-) to yield the double gene-targeted line (βARK1(-/-)βARKct). Offspring were genotyped by Southern blot analysis on DNA extracted from tail biopsies. Mice of either sex, 4–6 months of age were used and compared with wild-type litter mates. The animals in this study were handled according to approved protocols and the animal welfare regulations of the University of North Carolina at Chapel Hill and Duke University.

Hemodynamic Evaluation in Intact Anesthetized Mice—Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) and analyzed as described previously (8). Briefly, after endotracheal intubation and trachea were connected to an isolated perfusion apparatus, the heart was excised and the left atrium advanced through the mitral valve, and secured in the LV. Hemodynamic measurements were recorded at baseline and 45–60 seconds after injection of incremental doses of isopropenol. Doses of isopropenol were specifically chosen to maximize the contractile response but limit the increase in heart rate. Experiments were then terminated, hearts were rapidly excised, with individual chambers separated, weighed, and frozen in liquid N2 for later biochemical analysis. Ten sequential beats were averaged for each measurement.

Myocyte Isolation—Adult myocytes were isolated as described previously (20, 21). Following anesthesia, the heart was excised and the aorta was cannulated with a 20-gauge needle then mounted on the perfusion apparatus. The perfusion solution was composed of Joklik’s minimal essential medium containing (in mM) 113 NaCl, 4.7 KCl, 0.6 KH2PO4, 0.6 NaH2PO4, 1.2 MgSO4, 0.5 MgCl2, 10 HEPES, 20 d-glucose, 30 taurine, 2.0 carnitine, 2.0 creatine, and 20 μM Ca2+ at pH 7.4. The aorta was perfused for 2–3 min, then 150 units/ml of type-II collagenase ( Worthington) was added and perfused for 15 min. The temperature of perfusate was maintained at 34 °C and all solutions were continuously bubbled with 95% O2, 5% CO2. LV tissue was separated from the great vessels, atria and right ventricle, minced, and allowed to digest in perfusate for 15 min. The digested heart was filtered through 200 μm nylon mesh, placed in a conical tube, and spun at 100 rpm to allow viable myocytes to settle. Serial washes were used to remove nonviable myocytes and digestive enzymes until the concentration of Ca2+ was gradually increased to 1.8 mM in Joklik’s minimal essential medium. The operator was blinded to the genotype of the animals.

Evaluation of Myocyte Function—Myocytes were placed in a 0.5-mL chamber with 1.8 mM Ca2+ Tyrode’s solution at room temperature. Myocytes were visualized with a Nikon inverted microscope with a solid state CCD camera attached and displayed on a video monitor. Two platinum electrodes placed in the bathing fluid were connected to a stimulator to field stimulate the myocytes with a pulse duration of 5 ms and a frequency of 0.5 Hz. Myocyte cell edges were enhanced and processed with a video edge motion detection system (Crescent Electronics) at a sampling rate of 240 Hz. Recordings were performed under basal conditions and then 1–2 min after isoproterenol (10−6 M) administration. Calibrated myocyte lengths were converted from analog to digital on-line (MacLab) and stored on computer. All myocytes were studied within 1–2 h after myocyte isolation. Data from 5–8 consecutive contractions were averaged. Contractile parameters measured were: percent cell shortening (%CS) (calculated as percent change in myocyte length from rest (Lmax) to minimum length (Lmin)), rate of shortening (dL/dt), rate of relengthening (+dL/dt), and 7–15 myocytes from each heart were studied.

βARK Activity by Rhodopsin Phosphorylation—Myocardial extracts were prepared by homogenization of excised hearts in ice-cold lysing buffer (2 mL) (25 mM Tris-Cl (pH 7.5), 5 mM EDTA, 5 mM EGTA, 10 μg/mL leupeptin, 20 μg/mL aprotinin, and 1 mM phenylmethanesulfonyl fluoride) and centrifuged at 48,000 × g for 30 min. The supernatants that contain soluble kinases were concentrated using a Centricon-10 (Amicon) microconcentrator. Protein concentration was determined by the Bradford method. Concentrated cytosolic extracts (200 μg of protein) were incubated with rhodopsin-enriched rod outer segment membranes in reaction buffer (75 μL) containing 10 mM MgCl2, 20 mM Tris-Cl, 2 mM EDTA, 5 mM EGTA, and 0.1 mM ATP (containing [γ-32P]ATP) as described (9). Reactions were carried out in the absence and presence of purified Gαs (20 pmol) to maximally activate βARK (9). After incubating in white light for 15 min at room temperature, reactions were quenched with ice-cold lysis buffer (300 μL) and centrifuged for 15 min at 13,000 × g. Sedimented proteins were resuspended in 20 μL of protein-gel loading dye and electrophoresed through 12% SDS-polyacrylamide gels. Phosphorylated rhodopsin was visualized by autoradiography of dried polyacrylamide gels and quantified using a Molecular Dynamics PhosphorImager.

Statistical Analysis—Results are expressed as mean value ± S.E. To examine the effect of isopropenol on changes in hemodynamic parameters between wild-type controls and the two gene-targeted groups (βARK1(+/-) and βARK1(-/-)/βARKct), a 3 × 4 repeated measures analysis of variance (ANOVA) was used. To test for statistical difference in isolated cell contractile parameters and adenylyl cyclase activity, a one factor ANOVA was used. Post hoc analysis with regard to differences in mean values between groups was conducted with either a Newman-Keuls or Duncan test. A Student’s t test with Bonferroni correction for 3 comparisons was used to test for statistical difference in the chamber weight parameters. p < 0.05 was considered significant.

RESULTS

To determine whether altered levels of βARK1 influence myocardial growth in the adult mouse, left ventricular weight normalized for body weight (LV/BW) and tibia length was compared in wild type (n = 26), βARK1(+/-) (n = 19), and βARK1(-/-)/βARKct (n = 5) gene-targeted mice. No signifi-

G. Iaccarino, E. D. Tomhave, R. J. Lefkowitz, and W. J. Koch, submitted.
FIG. 2. In vivo assessment of LV contractile function in response to β-agonist stimulation. Cardiac catheterization was performed in intact anesthetized mice 4–6 months of age, using a 1.8-French high fidelity micromanometer. Parameters measured were LV systolic and end-diastolic pressure, the maximal and minimal first derivative of LV pressure (LV dP/dt max, LV dP/dt min), and heart rate. Four measured parameters are shown at baseline and after progressive doses of isoproterenol in wild-type (○) n = 26, and βARK1(+/−) (●) n = 19, and βARK1(+/−)/βARKct (▲) n = 9 mice. A, LV dP/dt max; B, LV dP/dt min; C, LV systolic pressure; D, heart rate. Data was analyzed with a repeated measures ANOVA and post hoc analysis by Newman-Keuls, *, p < 0.005 either βARK1(+/−) or βARK1(+/−)/βARKct versus wild type; †, p < 0.05 βARK1(+/−) versus βARK1(+/−)/βARKct. A significant between-group main effect in response to isoproterenol was found for LV dP/dt max (p < 0.0001) (A), LV dP/dt min (p < 0.0001) (B), LV systolic pressure (p < 0.0005) (C), and heart rate (p < 0.005) (D). The pattern of change between groups was statistically different for LV dP/dt max (p < 0.0001) (A) and heart rate (p < 0.0001) (D). Body weight was not significantly different between groups: wild type 27.6 ± 1.2 g; βARK1(+/−) 30.7 ± 1.1 g; βARK1(+/−)/βARKct 29.0 ± 1.5 g.

A significant difference was observed for any of the measured variables between the three groups (LV/BW; wild type 3.5 ± 0.1, βARK1(+/−) 3.7 ± 0.1, βARK1(+/−)/βARKct 3.6 ± 0.2, mg/g, p = not significant). In contrast to the embryonic lethal phenotype of the homozygous βARK1 knockout, the heterozygote mice developed normally and attained a similar body weight as wild-type adults. Similarly, we previously had not observed any differences in heart weight in the animals overexpressing the βARKct alone compared with wild type controls (7).

To assess the levels of myocardial βARK activity in the different gene-targeted mice, we prepared soluble myocardial extracts and carried out in vitro GRK phosphorylation assays using rhodopsin as a G protein-coupled receptor substrate. To address whether the βARKct is functional, we added purified Gβγ to the reactions. As shown in Fig. 1, Gβγ-stimulated βARK activity is decreased in a stepwise fashion with the βARK1(+/−)/βARKct animals having only ~25% of the wild-type myocardial βARK activity. Myocardial extracts from the βARK1(+/−) animals had 50% of the wild-type activity, which correlates to the 50% decrease in βARK1 protein we have previously described (9). This significant decrease in myocardial βARK activity in the double gene-targeted mice could also be demonstrated when expressing the data as fold-stimulation of Gβγ over basal rhodopsin phosphorylation activity (−Gβγ). In βARK1(+/−)/βARKct animals, Gβγ only stimulated activity by 2.24 ± 0.30-fold (n = 5) compared with 4.48 ± 0.93-fold (n = 6) for the wild-type animals (p < 0.05). Extracts from βARK1(+/−) hearts had similar values to wild-type (3.75 ± 0.88-fold, n = 6).

Because of the dependence of the βARK activity on Gβγ, as expected, in vitro βARK activity in the absence of Gβγ was equivalent between βARK1(+/−) extracts and βARK1(+/−)/βARKct extracts, which was ~50% of wild-type activity (data not shown).

We have previously reported that overexpression of the
acker tracings of myocytes isolated from a wild-type and a
eryol with the greatest level observed in the
mice. Contractile function was further and significantly aug-
infusion (7). To determine whether the level of
under basal conditions and in response to catecholamine
vivo after infusion of isoproterenol (Fig. 2). Although the isovolumic
zation was performed in intact anesthetized mice before and
b of myocardial
changes in cell length were measured in the same cell before and
during isoproterenol administration (10
M) 212.0
M/s)
max, was
rate were increased in the gene-targeted animals at baseline,
ARK1(−/−) or ARKct animals but not in the
ARK1(+/−) at baseline (Fig. 2B). As shown in Fig. 2, C and D, LV systolic pressure and heart
rate were increased in the gene-targeted groups at baseline, which was further potentiated with isoproterenol stimulation. This is particularly apparent for changes in heart rate (Fig. 2D).

Heart rate is a powerful determinant of myocardial contrac-
tility can be directly influenced by the level of

In vivo
assessment
contractility as measured by LV dP/dt
max. Taken together these data demonstrate that the level of βARK1 activity exerts tight con-
trol over the inotropic and chronotropic response to cate-
cholamine stimulation in the heart in vivo.

Since both loading conditions (LV end-diastolic pressure) and heart rate influence the in vivo measurement of myocardial
contractility as measured by LV dP/dt
max (22, 23), studies were performed on adult myocardial cells isolated from both of the
gene-targeted mouse strains. To determine whether a decrease in the level of βARK1 would affect myocyte contractility in the absence of potential confounding influences such as heart rate and mechanical loading, freshly isolated single adult
myocytes were obtained from normal, βARK1(+/−), and
ARK1(+/−)/βARKct gene-targeted mice followed by an
essment of the contractile properties.

Measurements of contractile parameters in unloaded isolated
adult cells were made in the absence and presence of isoproter-
enol (10−7 m) at a constant paced stimulation of 0.5 Hz. Adult
myocytes isolated from wild-type hearts responded to isoproter-
enol stimulation with a 12% increase in %CS and
rate of shortening (−dL/dt) without a change in diastolic cell
length (Fig. 4 and Table I). In contrast, adult myocytes isolated
βARK1(+/−) heterozygote animals showed an 18% increase in
−dL/dt, whereas myocytes from βARK1(+/−)/βARKct hearts showed an even greater increase in −dL/dt (29%) following isoproterenol administration (Table I and Fig. 4). Similarly, %CS
under baseline and isoproterenol conditions was progressively higher in myocytes isolated from the two gene-targeted mouse lines compared with wild-type cells (Table I). Overall cells isolated
βARK1(+/−) and βARK1(+/−)/βARKct mice had a
itively, increase in contractile parameters with isoproterenol compared with wild-type litter
mates (Table I). These data complement the in vivo assessment of contractile function and show that intrinsic myocyte contractility can be directly influenced by the level of βARK1.

**DISCUSSION**

The present study demonstrates that 1) βARK1(+/−) mice that are heterozygote for ablaiion of the βARK1 gene and have a
50% reduction in the level of βARK1 in the heart develop normally, 2) the level of chronotropy and inotropy in vivo can be modulated by the level of βARK1 expression, and 3) contractile function can be further enhanced through in vivo βARK1 inhibition by competing for Gβγ binding and endogenous βARK1 translocation and activation.

Complete disruption of the βARK1 gene in mice leads to a
lethal phenotype with no βARK1(−/−) embryos surviving beyond
geostationary day 15.5 (9). The finding that mice that are
heterozygous for the βARK1 deletion have no developmental abnormalities and develop into normal adults, suggests that there is a threshold level for βARK1 that allows for normal
ardiac development. Although the βARK1(+/−) mice grow
into normal adults, the functional consequence of reduced

**TABLE I**

| Contractile parameters in adult myocytes isolated from gene-targeted mice |
|-------------------------------------------------|------------------|-------------------|
|                                   | Wild type (n = 5) | βARK1(+/−) (n = 6) | βARK1(+/−)/βARKct (n = 5) |
|---------------------------------|------------------|-------------------|--------------------------|
| −dL/dt (μm/s)                  | −330.1 ± 13.0    | −369.9 ± 13.5     | −373.8 ± 20.8            |
| +dL/dt (μm/s)                  | 212.0 ± 16.3     | 240.2 ± 19.2      | 269.8 ± 22.9             |
| Maximum length (μm)            | 124.1 ± 2.7      | 124.4 ± 2.6       | 121.7 ± 2.7              |
| Minimum length (μm)            | 103.2 ± 3.0      | 100.8 ± 3.1       | 97.9 ± 2.6               |
| % CS                            | 17.1 ± 0.7       | 19.2 ± 0.9        | 19.7 ± 0.8              |
| a,b,c,d,e,f                      |                  |                   |                          |

a, b, c, d, e, f

"p < 0.05 βARK1(+/−) or βARK1(+/−)/βARKct vs. wild type.

"p < 0.05 βARK1(+/−)/βARKct vs. βARK1(+/−).

"p < 0.05 βARK1(+/−)/βARKct vs. wild type.

"p < 0.05 βARK1(+/−)/βARKct vs. βARK1(+/−).
βARK1 levels is a phenotype of decreased desensitization in the heart as shown by the enhanced contractile response to isoproterenol stimulation (Fig. 2).

βARK1 requires a membrane-targeting event prior to receptor phosphorylation that occurs through the interaction of membrane anchored G_pro, subunits and the carboxyl terminus of βARK1 (5). Preventing βARK1 translocation by competing for G_pro, binding in transgenic mice overexpressing a peptide inhibitor (βARKct) results in an in vivo phenotype of enhanced basal and agonist-stimulated contractility due to decreased receptor desensitization (7). The mating of these two gene-targeted mice (βARK1(+/-) and βARKct overexpression) results in a further enhancement of contractility and relaxation (Fig. 2). These data suggest that both the level of βARK1 expression and the active process of translocation and activation of βARK1, determine the degree of βAR desensitization and subsequent receptor-G protein coupling.

The isovolumic phase index (LV dp/dt max) is a sensitive measure of contractility. However, the level of contractility is significantly influenced by heart rate and loading conditions in particular, preload (23). In this regard, it has recently been shown that there is a linear relationship between heart rate and LV dp/dt_max (22). In the present study we show that the significantly enhanced contractile performance of mice with altered levels of βARK1, as measured by LV dp/dt_max is also associated with a modest increase in heart rate. To address this issue, we specifically assessed contractile parameters in single adult ventricular myocytes isolated from both the βARK1(+/-) and βARK1(+/-)/βARKct gene-targeted mice to determine whether the in vivo phenotype in these animals can be attributed to an intrinsic increase in myocyte contractility. The effect of heart rate on contractile function was eliminated by pacing cells at 0.5 Hz. As shown in Table I, a significant augmentation of contractility at a cellular level and confirm the dramatic beneficial effect of overexpression of a βARK1 inhibitor in a mouse model of heart failure (19).

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