Inhibition of receptor-localized PI3K preserves cardiac β-adrenergic receptor function and ameliorates pressure overload heart failure

Jeffrey J. Nienaber,1 Hideo Tachibana,1 Sathyamangla V. Naga Prasad,1 Giovanni Esposito,2 Dianqing Wu,3 Lan Mao,1 and Howard A. Rockman1

1Departments of Medicine, Cell Biology, and Genetics, Duke University Medical Center, Durham, North Carolina, USA
2Division of Cardiology, Federico II University, Naples, Italy
3University of Connecticut Health Center, Farmington, Connecticut, USA

β-Adrenergic receptor (βAR) downregulation and desensitization are hallmarks of the failing heart. However, whether abnormalities in βAR function are mechanistically linked to the cause of heart failure is not known. We hypothesized that downregulation of cardiac βARs can be prevented through inhibition of PI3K activity within the receptor complex, because PI3K is necessary for βAR internalization. Here we show that in genetically modified mice, disrupting the recruitment of PI3K to agonist-activated βARs in vivo prevents receptor downregulation in response to chronic catecholamine administration and ameliorates the development of heart failure with pressure overload. Disruption of PI3K/βAR colocalization is required to preserve βAR signaling, since deletion of a single PI3K isoform (PI3Kγ knockout) is insufficient to prevent the recruitment of other PI3K isoforms and subsequent βAR downregulation with catecholamine stress. These data demonstrate a specific role for receptor-localized PI3K in the regulation of βAR turnover and show that abnormalities in βAR function are associated with the development of heart failure. Thus, a strategy that blocks the membrane translocation of PI3K and leads to the inhibition of βAR-localized PI3K activity represents a novel therapeutic approach to restore normal βAR signaling and preserve cardiac function in the pressure overloaded failing heart.


Introduction

Heart failure is a syndrome characterized by depressed ventricular function, fluid retention, and increased mortality (1). Classic characteristics of the failing heart include a reduction in β-adrenergic receptor (βAR) number and diminished contractile response to catecholamine stimulation, due in part to activation of the sympathetic nervous system (2, 3). Treatment of heart failure patients with beta-blockers may reverse this process, and recent clinical and experimental data support the concept that normalizing βAR function can lead to improved cardiac function and prolonged survival (4–6). Despite these newer therapies, however, the mortality rate for patients with chronic heart failure remains high, indicating a need for novel strategies that are synergistic with current treatments.

βARs belong to the large family of G protein–coupled receptors (GPCRs) that relay their signals by coupling to G proteins and activating or inhibiting different effector molecules, such as an enzyme or ion channel (3, 7). Under conditions of heart failure there is a downregulation in the number of βARs and diminished contractile response to catecholamine stimulation, due in part to activation of the sympathetic nervous system (2, 3). Upon catecholamine binding to βARs, the heterotrimeric G proteins dissociate into Gα1 and Gβγ subunits. The Gα1 subunit is free to activate the effector adenyl cyclase to generate the second messenger cAMP and lead to an increase in heart rate and contractility. The termination of GPCR signals is initiated by phosphorylation of the agonist-occupied receptor primarily by the G protein–coupled receptor kinase (commonly known as βARK1), followed by the binding of arrestin proteins. Receptor phosphorylation and arrestin binding not only act to sterically inhibit further G protein activation, but is also the first step in the process of
receptor internalization and downregulation (7), a process that we have shown to require the presence of PI3K within the agonist-occupied receptor complex (8). PI3Ks are a conserved family of enzymes that catalyze the generation of D-3 phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P3) (8), which in turn regulate diverse cellular processes such as cell survival and proliferation, cytoskeletal rearrangements, receptor endocytosis, and cardiac contractility (9–11). Based on their structure and substrate specificity, PI3Ks can be divided into three classes (I, II, and III). The class I PI3Ks can be further subdivided into class Iα (activated by receptor tyrosine kinases, PI3Kα) and Iβ (activated by Gαs subunits of heterotrimeric G proteins, PI3Kγ). Recent studies have demonstrated important roles for both PI3Kα and PI3Kγ in cardiac growth and function (9, 12, 13). For instance, PI3Kγ is upregulated in response to in vivo pressure overload hypertrophy in the heart (14), and the absence or inhibition of PI3Kγ leads to enhanced cardiac contractility (9, 12). Moreover, overexpression of PI3Kα mutants in hearts of transgenic mice leads to changes in the size of cardiomyocytes, indicating this isoform may be important for cell growth in the heart (13). Whether PI3K plays a role in the development of heart failure through its interaction with βARs is unknown.

PI3K binds to βARK1 through a helical domain known as the phosphoinositide kinase domain (PIK), which is conserved among all PI3K isoforms. Upon agonist stimulation, βARK1 mediates the translocation of PI3K to βARs, allowing for the generation of phosphatidylinositol-3,4,5-tri-phosphate (PtdIns-3,4,5-P3) (8, 10). The local generation of PtdIns-3,4,5-P3 within the receptor complex functions to enhance the recruitment of a number of phosphoinositide-binding endocytic proteins such as β-arrestin and AP-2, essential for βAR internalization (8, 15). Furthermore, in cell culture systems, overexpression of a catalytically inactive PI3K or PIK domain that disrupts the interaction between PI3K and βARK1 by displacing both class Iα and Iβ isoforms blocks agonist-stimulated βAR internalization (8, 10).

Since βARK1-mediated localization of PI3K is required for internalization of βARs, we postulated that under conditions of excess catecholamines, similar to that found in chronic human heart failure, PI3K would play an important role in the chronic downregulation of βARs. To test this hypothesis that βAR downregulation and cardiac dysfunction could be prevented in vivo by blocking the βARK1-mediated recruitment of active PI3K, we studied mice with cardiac-specific overexpression of a catalytically inactive mutant of PI3Kγ (PI3Kγinact) and PI3Kγ knockout mice (PI3KγKO) following exposure to chronic catecholamine administration or pressure overload–induced heart failure.

Methods

Generation of transgenic mice. The pCMV-PI3Kp110γ-hemagglutinin (pCMV-PI3Kp110γ-HA) mutant (PI3Kγinact) (Δ942-981, a deletion in the ATP-binding site) was a generous gift from Charles S. Abrams (Department of Medicine, University of Pennsylvania Medical School, Philadelphia, Pennsylvania, USA) (16). The cDNA insert of PI3Kγinact was amplified using a Pfu high-fidelity enzyme (Stratagene, La Jolla, California, USA) with a 5′ primer (5′-TGGGATCCCGCCCACGAAAAGAC-3′) and a 3′ primer (5′-ACCCGGATCTTTAGGTTGTAAT-3′) containing Clai for subcloning, followed by Kozak consensus sequence and 3′-primer (5′-ACCCGGATCTTTAGGTTGTAAT-3′) with a Clai site for subcloning. The PCR product was subcloned directly into a vector downstream of the αMyHC gene promoter and upstream of an HA epitope and an SV40 polyadenylation site. The subcloned cDNA was sequenced and digested with restriction enzymes Xhol and NotI to check for orientation. Transgenic founders were identified by Southern blot analysis of tail DNA using the SV40 poly(A) as a probe. Transgenic founder mice were backcrossed into both DBA and C57BL/6 backgrounds for nine generations. The DBA background PI3Kγinact transgenic mice was used for the osmotic pump study while C57BL/6 was used in the transverse aortic constriction (TAC) studies. The PI3Kγ knockout mice were a generous gift from Dianqing Wu (University of Connecticut Health Center), and their method of generation has been published previously (17). The background of the PI3Kγ knockout mice was 129/B6, and 129/B6 mice were used as controls. Animals were handled according to the approved protocols and animal welfare regulations of the Institutional Review Board at Duke University Medical Center.

Echocardiography. Echocardiography was performed on conscious mice at 3–4 months of age with an HDI 5000 echocardiograph (ATL, Bothell, Washington, USA) as described previously (18). Mini-osmotic pump implantation. Mini-osmotic pumps were implanted as described previously (19). Isoproterenol was dissolved in 0.002% ascorbic acid, and pumps (Alzet model 1007D; DURECT Corp., Cupertino, California, USA) were filled to deliver at a rate of 3 mg/kg/d over a period of 7 days. In control mice pumps that delivered vehicle (0.002% ascorbic acid) were implanted.

In vivo pressure overload. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (2.5 mg/kg), and TAC was performed as described previously (18). Twelve weeks after surgery, the transstenotic pressure gradient was assessed by recording simultaneous measurement of right carotid and left axillary arterial pressures.

Hemodynamic evaluation in mice. Hemodynamic evaluation was performed as described previously (20). Hemodynamic measurements were recorded at baseline and 45 seconds after infusion of incremental doses of isoproterenol (50, 500, and 1,000 pg). Hemodynamic measurements were analyzed by SONOVIEW software (Sonometrics Corp., London, Ontario, Canada).

Membrane fractionation, lipid kinase assay, βARK radioligand binding and adenyl cyclase activity. Total PI3K and PI3Kγ assays from the left ventricles (LV) flash-frozen
in liquid N$_2$ were carried out as described previously (10). Membrane fractions were prepared as described previously (18). For the βARK1-associated PI3K assay, 500 µg of membrane fraction was used for immunoprecipitation with the C5/1 mAb directed against βARK1 (10). To directly measure the generation of PtdIns-3,4,5-tri-phosphate (PIP3), in vitro lipid kinase assays were performed using the substrate PtdIns-4,5-P$_2$, which can be used by class I PI3Ks to generate PtdIns-3,4,5-P$_3$. The lipids were extracted with chloroform/methanol (ratio of 1:1), and organic phase was spotted on TLC plates and resolved chromatographically with 2N glacial acetic acid/1-propanol (ratio of 35:65). Dried plates were exposed, and autoradiographic signals were quantitated using Bio-Rad Phosphorimager. Receptor binding with 25 µg of the membrane fraction was performed as described previously (18) using βAR ligand ([125I] cyanopindolol, 250 pM). All assays were performed in triplicate, and receptor density (in femtomoles) was nor-

**Figure 1**

Generation of transgenic mice with decreased cardiac βAR-localized PI3K activity. (a) Diagram of the transgene construct containing the PI3Kγinact cDNA (upper panel). Southern (10 µg of DNA) and Western blots (100 µg of cytosolic extract) from WT and three transgenic founder lines using SV-40 probe and anti-PI3KγAb, respectively (lower panel). (b) and (c) Basal levels of total PI3K (b) and PI3Kγ (c) activity in the LV relative to WT (n = 9, WT and transgenic lines); 2 mg of cytosolic extract was used for immunoprecipitation with PI3K Ab. *P < 0.05 versus WT; †P < 0.01 versus WT. (d) Basal βARK1-associated PI3K activity in the LV of WT (n = 6) and 180-fold overexpressing transgenic mice (n = 6); 4 mg of cytosolic extract was used for immunoprecipitation with βARK1 mAb. (e) Total PI3K, PI3Kγ, and βARK1-associated PI3K activity in the left LVs measured by their ability to phosphorylate PtdIns-4,5-P$_2$ to PtdIns-3,4,5-P$_3$. (f) Immunoblotting for PI3Kα or PI3Kγ following immunoprecipitation with βARK1 mAb from clarified myocardial lysates (3 mg). *P < 0.001 versus WT. IP, immunoprecipitation; αMHC, alpha myosin heavy chain promoter; K, Kozak sequence; C2, similar to type II C2 domain found in phospholipase Cδ1; X, catalytically inactive; ABR, adaptor binding region; RBD, ras-binding domain; HA, hemagglutinin epitope tag; PIP, phosphatidylinositol mono-phosphate; PIP2, phosphatidylinositol bis-phosphate; Ori, origin; Cont (plus control), immunoprecipitation of PI3Kα or PI3Kγ with its respective Ab and immunoblotted for PI3Kα or PI3Kγ; βARK, β-adrenergic receptor kinase.
alized to milligrams of membrane protein. Adenylyl cyclase assays were performed as described previously (18) using 20 μg of the membrane fraction. Generated cAMP was quantified using a liquid scintillation counter (MINAXIβ-4000).

βARK1 activity by rhodopsin phosphorylation. Membrane (50 μg) and cytosolic fraction (150 μg) were incubated with rhodopsin-enriched rod outer segments with 10 mM MgCl2 and 100 μM ATP containing [γ-32P] ATP. The reactions were incubated in white light for 15 minutes, stopped, resolved by SDS-PAGE gel, and autoradiography was carried out as described previously (18).

Immunoblotting. Immunoblotting was done as described previously (18). Immunoprecipitating Ab’s were added to 500 µg of membrane fraction. Ab’s to phospho-protein kinase B (pPKB) and phosphoglycogen synthase kinase (pGSK) were used for blotting at 1:1,000, anti-hemagglutinin (PI3KYγinact-HA) at 1:500, and 1:10,000 for βARK1. Detection was carried out using ECL (Amersham Biosciences Corp., Piscataway, New Jersey, USA), and bands were quantified with densitometry.

MAPK. MAPK activities were assessed from clarified LV extracts as the capacity of immunoprecipitated extracellular signal-regulated kinase-p42/extracellular signal-regulated kinase-4 (ERK-p42/ERK-p44), p38, p38β, and JNK-p46/JNK3 MAPK to phosphorylate in vitro substrates (myelin basic protein or GST-JUN) as described previously (18).

Survival studies. Survival data were analyzed by using a Kaplan-Meier survival analysis with a log-rank method of statistics. Survival analysis excluded mice that died within 3 days after surgery (TAC) and mice that had a pressure gradient of less than 15 mmHg for both WT and PI3KYγinact mice.

Statistical analysis. Data are expressed as mean plus or minus SEM. Two-way repeated-measures ANOVA was used to evaluate hemodynamic measurement under basal and isoproterenol treatment and for analysis of cardiac function after TAC. Post-hoc analysis was performed with a Scheffé test. Two-sample comparisons were performed with Student’s t tests. The Student’s t test with Bonferroni correction for multiple comparisons was used to evaluate data from the isoproterenol time course. For all analysis, a value of P less than 0.05 was considered significant.

Results

Mice overexpressing PI3KYγinact have decreased basal PI3K and βARK1-associated PI3K activity. We generated PI3KYγinact mice by subcloning PI3KYγinact downstream of the αMHC promoter (Figure 1a, upper panel), which is expressed in cardiomyocytes (21). Three founders (numbers 64, 71, and 61) were generated, characterized by 20-, 90- and 180-fold overexpression of the mutant protein relative to WT (Figure 1a, lower panel). Robust total PI3K activity in WT mice was observed without a significant reduction in activity among the 20- and 90-fold overexpressing PI3KYγinact transgenic mice. The 180-fold overexpressing PI3KYγinact mice showed a 45% reduction in total PI3K compared with the WT (Figure 1b).

Since we have previously shown a direct interaction between βARK1 and PI3K (8, 10), we used the 180-fold overexpressing mice to determine whether the PI3KYγinact transgenic mice had decreased βARK1-associated PI3K activity by immunoprecipitating βARK1 from ventricular lysates and assaying for PI3K activity. Importantly, there was a marked reduction in βARK1-associated PI3K activity in PI3KYγinact hearts compared with WT (Figure 1d). We next tested the ability of WT and PI3KYγinact transgenic mice (180-fold) to generate PI3P,

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Physiologic parameters in WT and PI3KYγinact (TG) lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n = 15)</td>
</tr>
<tr>
<td>Morphometric</td>
<td></td>
</tr>
<tr>
<td>BW, g</td>
<td>27.5 ± 1.2</td>
</tr>
<tr>
<td>Tibial length, mm</td>
<td>17.54 ± 0.09</td>
</tr>
<tr>
<td>RV weight, mg</td>
<td>28.57 ± 1.61</td>
</tr>
<tr>
<td>LV weight, mg</td>
<td>96.97 ± 4.43</td>
</tr>
<tr>
<td>LVW/BW, mg/g</td>
<td>3.54 ± 0.08</td>
</tr>
<tr>
<td>LV/tibial length, mg/mm</td>
<td>5.53 ± 0.25</td>
</tr>
<tr>
<td>Echocardiographic (conscious) (n = 10)</td>
<td></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>613 ± 13</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>2.99 ± 0.06</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>1.48 ± 0.04</td>
</tr>
<tr>
<td>FS, %</td>
<td>51 ± 1</td>
</tr>
<tr>
<td>SEPh, mm</td>
<td>1.06 ± 0.04</td>
</tr>
<tr>
<td>PWth, mm</td>
<td>1.03 ± 0.06</td>
</tr>
<tr>
<td>Mean Vcfc, circ/s</td>
<td>3.96 ± 0.15</td>
</tr>
<tr>
<td>Hemodynamic (anesthetized) (n = 13)</td>
<td></td>
</tr>
<tr>
<td>LV dp/dtmax, mmHg/s</td>
<td>7,954 ± 731</td>
</tr>
<tr>
<td>LV dp/dtmin, mmHg/s</td>
<td>6,038 ± 427</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>5.5 ± 0.6</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>90.4 ± 3.3</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>415 ± 7</td>
</tr>
</tbody>
</table>

TG, transgenic; RV, right ventricle; LVW, LV weight; bpm, heart beats per minute; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; FS, fractional shortening (LVEDD – LVESD)/LVEDD; SEPh, septal wall thickness; PWth, posterior wall thickness; circ/s, circumference/second; mean Vcfc; heart rate-corrected mean velocity of circumferential fiber shortening calculated as FS divided by ejection time multiplied by the square root of the R-R interval; LV dp/dtmax and dp/dtmin, maximum and minimum first derivatives of the LV pressure; LVEDP, LV end-diastolic pressure; LVSP, LV systolic pressure.
the physiologically relevant phospholipid, using the specific class I PI3K substrate PtdIns-4,5-P2. Robust total PI3K and PI3Kγ activity was observed in the WT mice, while generation of PIP3 was attenuated in the PI3Kγ inact transgenic mice (Figure 1e). Importantly, reduced generation of PIP3 was also observed when βARK1 was immunoprecipitated and assayed for PI3K activity in the PI3Kγ inact mice (Figure 1e). To directly assess the association of PI3K with βARK1 in the heart, βARK1 was immunoprecipitated from myocardial lysates and blotted for the coimmunoprecipitating PI3K isoform. PI3Kγ interacts with βARK1 in the WT mice, and this interaction is increased in the PI3Kγ inact transgenic mice due to its association with the overexpressed PI3Kγ inact transgene (Figure 1f). In contrast, low levels of PI3Kα are found associated with βARK1 in the heart (Figure 1f).

To determine whether reduced βARK1-associated PI3K activity would alter the physiologic phenotype of PI3Kγ inact-overexpressing mice do not undergo βAR desensitization or downregulation under conditions of chronic catecholamine administration. (a) In vivo hemodynamic studies showing βAR responsiveness as monitored by the increase in left ventricular contractility (LV dP/dt max) in WT and PI3Kγ inact mice following 7 days of ISO (closed circles: n = 9; closed squares: n = 7) or vehicle (open circles: n = 13; open squares: n = 8) treatment. *P < 0.0001 (two-way ANOVA) WT ISO versus PI3Kγ inact ISO. (b) βARK1-associated PI3K activity in WT and PI3Kγ inact ventricles (500 µg of membrane fraction) following 7 days of ISO (n = 6) or vehicle treatment (n = 5–6). Values are expressed relative to vehicle-treated WT. Immunoblotting the HA-epitope tag (PI3Kγ inact) following immunoprecipitation with βARK1 from 500 µg of membrane fraction (upper immunoblot, IB: HA). *P < 0.001 ISO 7 days versus vehicle. (c) In vitro myocardial βARK1 activity measured by rhodopsin (Rho) phosphorylation in WT and PI3Kγ inact mice following 7 days of ISO or vehicle treatment. (d) βAR density among WT and PI3Kγ inact mice following 7 days of ISO administration (n = 6) or vehicle treatment (n = 5–6). *P < 0.05 ISO 7 days versus vehicle. (e) Basal (white bars) and in vitro ISO-stimulated (black bars) adenylyl cyclase activity in WT and PI3Kγ inact mice following 7 days of ISO (n = 6) or vehicle treatment (n = 5–6). Adenylyl cyclase activity upon NaF stimulation: 257 ± 21 pmol/mg/min and 244 ± 18 pmol/mg/min for vehicle and ISO 7-day treatment, respectively, in WT; 242 ± 12 pmol/mg/min and 239 ± 24 pmol/mg/min for vehicle and ISO 7-day treatment, respectively, in PI3Kγ inact mice. *P < 0.002 ISO versus basal. βARK1 (C5/1) mAb is directed against the catalytic site of βARK1 blocking its enzymatic activity.
contrast, treated PI3Kγ\textsuperscript{inact} mice showed normal contractile responses to catecholamines, indicating normal β\textsubscript{AR} sensitivity. Chronic ISO treatment resulted in a similar 20% increase in the LVW/BW ratios of both WT and transgenic mice (WT: vehicle 3.5 ± 0.1 mg/g, ISO 4.3 ± 0.1 mg/g; PI3Kγ\textsuperscript{inact}: vehicle 3.4 ± 0.1 mg/g, ISO 4.1 ± 0.1 mg/g, \(P = \text{NS}\)). Thus, overexpression of PI3Kγ\textsuperscript{inact} in the heart preserves β\textsubscript{AR} sensitivity under conditions of excess catecholamine levels, without affecting growth responses.

To determine whether chronic ISO administration reduces βARK1-associated PI3K activity in myocardial membranes, WT and PI3Kγ\textsuperscript{inact} mice were treated with ISO for 7 days. A robust, 2.6-fold increase in βARK1-associated PI3K activity was observed in WT mice following chronic ISO treatment (Figure 2b). This effect was completely abolished in the transgenic mice overexpressing PI3Kγ\textsuperscript{inact} (Figure 2b), despite the fact that PI3Kγ protein was recruited to the plasma membrane (Figure 2b; immunoblotting [IB]: HA). Cytosolic βARK1 expression was also increased with chronic ISO treatment in the hearts of both WT and PI3Kγ\textsuperscript{inact} mice under basal conditions, we assessed cardiac function by echocardiography and cardiac catheterization. No differences were found between WT and any of the PI3Kγ\textsuperscript{inact} lines for LV chamber dimensions, fractional shortening, heart rate, or in the LV weight/body weight (LVW/BW) ratios. Furthermore, cardiac catheterization of the WT and 180-fold overexpressing transgenic mice revealed no differences in contractile function (LV \(dP/dt_{\text{max}}\)) or other hemodynamic parameters (Table 1). These data suggest that although a high level of PI3Kγ\textsuperscript{inact} overexpression leads to a significant reduction in βARK1-associated PI3K activity, it does not result in any physiologic, morphometric, or hemodynamic changes under unstressed basal conditions (Table 1).

**Figure 3**

βARK1-mediated PI3K recruitment leads to desensitization and downregulation of β\textsubscript{AR}s. (a) βARK1-associated PI3K activity in WT and PI3Kγ\textsuperscript{inact} ventricles (500 µg of membrane fraction) following 12 hours, 24 hours, 3 days, and 5 days of ISO administration. (b) In vitro ISO-stimulated adenyl cyclase activity represented as the fold increase over basal level in WT (white bars) and PI3Kγ\textsuperscript{inact} (black bars) mice following 12 hours, 24 hours, 3 days, and 5 days of chronic ISO administration. (c) β\textsubscript{AR} density among WT and PI3Kγ\textsuperscript{inact} mice following 12 hours, 24 hours, 3 days, and 5 days of ISO infusion. (d) βARK1-associated PI3K activity measured by the ability to in vitro phosphorylate PtdIns-4,5-P\textsubscript{2}, as a substrate to generate PtdIns-3,4,5-P\textsubscript{3} in WT and PI3Kγ\textsuperscript{inact} ventricles (500 µg of membrane fraction) following 5 days of ISO administration. *\(P < 0.05\) WT ISO treated versus WT vehicle. †\(P < 0.05\) WT ISO versus PI3Kγ\textsuperscript{inact} ISO at same time point. Sub, substrate.
hearts (data not shown). Therefore, overexpression of PI3Kγ
inact displaces active endogenous PI3K from βARK1, leading to the translocation of inactive PI3K by βARK1 to the myocardial membrane under conditions of agonist stimulation.

Translocation of βARK1 depends on dissociated Gβγ subunits from heterotrimeric G proteins (22). Since PI3Kγ binds Gβγ subunits, we tested whether overexpression of PI3Kγ
inact alters the membrane recruitment of βARK1. Membrane and cytosolic extracts from WT and PI3Kγ
inact transgenic hearts were measured for their capacity to phosphorylate the GPCR rhodopsin. Membrane extracts from chronic ISO-treated hearts showed a similar increase in βARK1 activity (Figure 2c) that was abolished by preincubation with βARK1 mAb (anti-βARK1/βARK2-C5/1) (Figure 2c) (23). These data demonstrate that overexpression of PI3Kγ
inact does not alter the function or recruitment of βARK1 to myocardial membranes.

To determine whether inhibition of locally generated PtdIns-3,4,5-P3 within the plasma membrane diminishes the degree of βAR downregulation and desensitization that occurs with chronic ISO treatment, we measured βAR density and adenylyl cyclase activity in crude myocardial membrane preparations from WT and transgenic mouse hearts after 7 days of chronic ISO treatment. βAR density was reduced 33% in chronic ISO-treated WT hearts compared with vehicle-treated WT hearts (Figure 2d). In contrast, no significant decrease in βAR density occurred in the ISO-treated PI3Kγ
inact hearts compared with vehicle-treated PI3Kγ
inact hearts.
hearts (Figure 2d). Furthermore, overexpression of PI3Kγ\textsubscript{γ\textsubscript{inact}} completely preserved adenylyl cyclase activity following chronic ISO treatment compared with WT (Figure 2e). Taken together, these data show that under conditions of high levels of circulating catecholamines, overexpression of PI3Kγ\textsubscript{γ\textsubscript{inact}} in the heart preserves in vivo βAR responsiveness by preventing the downregulation and desensitization of myocardial βARs.

To determine the time course of βARK1-mediated PI3K recruitment, receptor desensitization, and downregulation, WT and PI3Kγ\textsubscript{γ\textsubscript{inact}} transgenic mice were treated with isoproterenol by mini-osmotic pump for 12 hours, 24 hours, 3 days, and 5 days. Significant βARK1-associated PI3K activity was observed in membrane fractions of WT mice within 12 hours of ISO and was sustained throughout the entire time course of ISO administration (Figure 3a). In contrast, βARK1-mediated PI3K recruitment was prevented in the PI3Kγ\textsubscript{γ\textsubscript{inact}} transgenic mice over the same time period (Figure 3a). Vehicle treatment had no effect on the recruitment of PI3K (Figure 3a and Figure 2b). βAR receptor desensitization as measured by diminished ISO-stimulated membrane adenylyl cyclase activity also occurred within 12 hours of ISO administration (Figure 3b), which was also prevented in the PI3Kγ\textsubscript{γ\textsubscript{inact}} transgenic mice (Figure 3b). As opposed to the early events of PI3K recruitment and βAR desensitization, downregulation of βARs did not occur until 3 days of ISO administration and was significantly reduced 30% upon 5 days of chronic catecholamine administration (Figure 3c). Importantly, no significant changes in βAR density occurred in the PI3Kγ\textsubscript{γ\textsubscript{inact}} transgenic mice throughout the entire ISO administration time course (Figure 3c).

The ability of βARK1-associated PI3K to generate PIP3 was measured using the in vitro substrate PtdIns-4,5-P\textsubscript{2} from the membranes of 5-day ISO-treated WT and PI3Kγ\textsubscript{γ\textsubscript{inact}} mice. Robust PIP3 production was observed with the βARK1 immunoprecipitates from the WT mice, which was completely blocked in the PI3Kγ\textsubscript{γ\textsubscript{inact}} transgenic mice (Figure 3d). These data show that overexpression of PI3Kγ\textsubscript{γ\textsubscript{inact}} transgene displaces endogenous PI3K from βARK1 and that βARK1-mediated recruitment of PI3K and receptor desensitization occurs early with agonist stimulation followed by receptor downregulation.

PI3Kγ\textsubscript{γ\textsubscript{inact}} overexpression displaces all endogenous interacting PI3K isoforms from βARK1. An important issue is whether the preservation of βAR functions with chronic ISO in the transgenic mice occurred because of the displacement of endogenous PI3K from the βARK1/PI3K complex, as we postulate, or due to a generalized reduction in cellular PI3Kγ activity. To test the latter possibility, we used PI3Kγ knockout (PI3Kγ-KO) mice that were homozygous for the PI3Kγ null allele. Mini-osmotic pumps containing ISO or vehicle were implanted in both WT and PI3Kγ-KO mice and hemodynamic studies performed 7 days later. ISO induced a brisk increase in LV dp/dt\textsubscript{max} in both the vehicle-treated WT and PI3Kγ-KO mice (Figure 4a), whereas in vivo βAR responsiveness was severely attenuated in chronically ISO-treated PI3Kγ-KO and WT mice. The difference in basal LV dp/dt\textsubscript{max} for WT control of PI3Kγ-KO mice and WT controls of PI3Kγ\textsubscript{γ\textsubscript{inact}} transgenic mice is likely attributed to the different genetic backgrounds of these mice and is consistent with our previous studies (24, 25). Importantly, a similar response to ISO is observed for the two inbred WT strains with a twofold increase in LV dp/dt\textsubscript{max} at maximal doses of ISO.

We further evaluated βAR density and adenylyl cyclase activity in crude myocardial membrane preparations from WT and PI3Kγ-KO mice, and βAR density (Figure 5).

**Figure 5**
Cardiac-specific overexpression of PI3Kγ\textsubscript{γ\textsubscript{inact}} does not alter downstream PI3K signaling. (a) JNK, ERK, p38, and p38β MAPK activities from 1 mg left ventricular myocardial extracts of WT and PI3Kγ\textsubscript{γ\textsubscript{inact}} mice under unstimulated conditions. Myocardial extracts (100 μg) from WT and PI3Kγ\textsubscript{γ\textsubscript{inact}} mice immunoblotted for pPKB and pGSK. White bars, WT; black bars, PI3Kγ\textsubscript{γ\textsubscript{inact}} KO. *P < 0.01 PI3Kγ\textsubscript{γ\textsubscript{inact}} Versus WT. (b) Myocardial extracts from WT and PI3Kγ\textsubscript{γ\textsubscript{inact}} mice immunoblotted for pPKB and pGSK. (c and d) PKB, GSK, and phospho-ERK (pERK) immunoblots from 100 μg of myocardial extract of WT and PI3Kγ\textsubscript{γ\textsubscript{inact}} mice following 7 days of ISO treatment (c) and upon insulin stimulation (d). (e) Hypertrophic response to pressure overload induced by TAC measured as a ratio of left ventricular weight (LVW/BW) plotted against the systolic pressure gradient produced by transverse aortic constriction for each WT (open circles: n = 15) and PI3Kγ\textsubscript{γ\textsubscript{inact}} (closed circles: n = 19) mouse. Mean pressure gradient between the groups was similar, WT 83.3 ± 6.1 and PI3Kγ\textsubscript{γ\textsubscript{inact}} 77.0 ± 3.6 mmHg. JNK, c-Jun NH\textsubscript{2}-terminal kinase; ERK, extracellular signal-regulated kinase; PKB, protein kinase B; GSK, glycogen synthase kinase B; MBP, myelin basic protein.
ty was equally reduced approximately 40% in chronic ISO-treated WT and PI3Kγ-KO hearts (Figure 4b) compared with the vehicle-treated hearts. Despite the increase in basal adenylyl cyclase activity in the PI3Kγ-KO mice (Figure 4c), as previously described (9), chronic ISO treatment resulted in marked desensitization to an extent similar to the WT mice (Figure 4c). Taken together, these data show that under conditions of high levels of circulating catecholamines absence of PI3Kγ does not prevent βAR downregulation or receptor desensitization, in marked contrast to the results obtained in the PI3Kγ inact transgenic mice.

The above PI3Kγ-KO studies suggest that βARK1 may be interacting with other PI3K isoforms to promote βAR internalization, a finding we have shown previously in vitro for PI3Kα (10). To demonstrate that βARK1 interacts with other PI3K isoforms in the heart, total and βARK1-associated PI3K activity was measured from the myocardial lysates of WT and PI3Kγ-KO mice. PI3K activity was not detectable in the knockout mice, confirming the null phenotype (data not shown), while total PI3K activity was markedly reduced (Figure 4d).

We found the level of βARK1-associated PI3K activity in the hearts of PI3Kγ-KO mice, however, to be identical to that of the WT hearts (Figure 4, e and f), however, and this lipid kinase activity was wortmannin sensitive (Figure 4f). To test directly for the interacting PI3K isoform with βARK1 in the PI3Kγ-KO mice, βARK1 was immunoprecipitated from clarified myocardial lysates and blotted for both PI3Kα and PI3Kγ. As shown in Figure 4g, PI3Kα is the only isoform that coimmunoprecipitates with βARK1 in the PI3Kγ-KO mice. Taken together, these data show that interrupting the βARK1/PI3K interaction in the heart in vivo preserves βAR function in response to chronically elevated levels of circulating catecholamines. These data also show that in the absence of PI3Kγ, βARK1 can interact with other PI3K isoform(s) in vivo to promote downregulation and desensitization of βARs.

Cardiac-specific overexpression of PI3Kγ inact does not alter downstream PI3K signaling or development of cardiac hypertrophy. Since PI3K plays an important role in regulating cellular signaling, we wanted to exclude the possibility that overexpression of PI3Kγ inact altered the activity of

Figure 6
PI3Kγ inact overexpression delays development of cardiac failure following chronic pressure overload induced by TAC. (a) Percentage of change in LV end diastolic dimension, LV end systolic dimension, and fractional shortening in WT (n = 22) and PI3Kγ inact (n = 23) mice measured by serial echocardiography at indicated time intervals after TAC. *p < 0.05 or †p < 0.01 for PI3Kγ inact versus WT at the same point. (b) Representative serial echocardiography in conscious WT and PI3Kγ inact mice with chronic pressure overload. (c) Kaplan-Meier survival analysis in WT (n = 46) and PI3Kγ inact mice (n = 48) after surgery (TAC). P < 0.007, PI3Kγ inact TAC versus WT TAC. (d) βARK1-associated PI3K activity in membrane fractions from hearts of WT and PI3Kγ inact mice (500 µg) (upper). Immunoblotting for the HA-epitope (PI3Kγ inact) following immunoprecipitation with βARK1 (lower).
downstream signaling pathways. Under unstimulated conditions, no effect in activity was observed for all the three major MAPK pathways (ERK, JNK, and p38/p38β) between the PI3Kγ-inact mice and their WT littermate controls (Figure 5a). While PI3Kγ-inact mice showed a significant decrease in pPKB, this decrease did not change the phosphorylation status of the immediate downstream enzyme glycogen synthase kinase (GSK3) (Figure 5a). In contrast to the findings in the PI3Kγ-inact mice, both pPKB and pGSK were reduced in the PI3Kγ-KO mice in the unstimulated basal state (Figure 5b). We further determined whether overexpression of PI3Kγ-inact transgene in the mice would affect immediate downstream signaling molecules like PKB, GSK3, and ERK following either GPCR or growth factor stimulation. Chronic ISO administration for 7 days showed a similar increase in both pPKB and pERK in the PI3Kγ-inact mice compared with the WT and had no effect on the level of pGSK3 (Figure 5c). Moreover, acute insulin administration led to significant increase of pPKB and pGSK levels in the PI3Kγ-inact transgenic mice, also similar to the increase observed in the hearts of WT mice (Figure 5d). These studies, taken together, show that overexpression of PI3Kγ-inact transgene does not interfere with the downstream signaling of GPCR or receptor tyrosine kinases.

Since acute agonist stimulation was not altered in PI3Kγ-inact mice, we wanted to determine whether overexpression of the PI3Kγ-inact transgene alters the response of the heart to a pleiotropic stimulus such as pressure overload–induced cardiac hypertrophy. The hypertrophic response after 7 days of pressure overload was measured as the index of LVW/BW and plotted against the systolic pressure gradient. Importantly, LVW/BW for individual WT and PI3Kγ-inact mice across a wide range of systolic pressure gradients was not different, indicating intact hypertrophic signaling pathways in the PI3Kγ-inact transgenic mice (Figure 5e). As a group there was a similar 150% increase in LV/BW after 7 days of TAC (pre-TAC WT, 3.35 ± 0.52 mg/g, and PI3Kγ-inact, 3.31 ± 0.46 mg/g, to post-TAC WT, 5.33 ± 1.00 mg/g, and PI3Kγ-inact, 4.83 ± 0.69 mg/g). Taken together, these data show that overexpression of PI3Kγ-inact does not inhibit the activation of downstream signaling pathways in the heart in response to multiple extracellular stimuli.

Cardiac-specific overexpression of PI3Kγ-inact preserves cardiac function under conditions of chronic pressure overload. PI3Kγ-inact mice and their WT littermates underwent TAC and were subsequently followed for 12 weeks. Serial echocardiography showed progressive LV enlargement and deterioration in cardiac function in the WT littermates (Figures 6, a and b) (Table 2). In contrast, PI3Kγ-inact overexpression significantly delayed the development of cardiac dysfunction and dilatation over the same time period following chronic pressure overload (Figures 6, a and b). Furthermore, survival of PI3Kγ-inact mice after TAC was significantly greater compared with the WT mice (mean survival PI3Kγ-inact 68 ± 4 days and WT 52 ± 5 days, P < 0.007; Figure 6c), consistent with amelioration of the cardiac phenotype. Transgenic and WT littermate mice used for studies in Table 1 were inbred on a DBA background, while mice used for TAC studies (Table 2) were inbred on a C57BL/6 background. Small differences in basal percentage of fractional shortening between Table 1 and Table 2 are likely attributed to differences in the genetic background.

Because PI3Kγ is recruited to the membrane by βARK1, we determined βARK1-associated PI3K activity in the membrane fractions of sham and TAC hearts. The increase in the βARK1-associated PI3K activity in the TAC WT hearts was abolished in the TAC PI3Kγ-inact mice (Figure 6d) and was replaced by inactive PI3Kγ-inact transgene (Figure 6d; IB: HA). Importantly, blocking the recruitment of active PI3K to the membrane prevented the downregulation of βARs in the cardiac membranes of TAC PI3Kγ-inact mice in contrast with the TAC WT

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>4 Weeks</th>
<th>8 Weeks</th>
<th>12 Weeks</th>
<th>Before</th>
<th>4 Weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>668 ± 6</td>
<td>649 ± 5</td>
<td>651 ± 6</td>
<td>655 ± 8</td>
<td>665 ± 6</td>
<td>657 ± 6</td>
<td>647 ± 9</td>
<td>647 ± 9</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.01 ± 0.05</td>
<td>3.40 ± 0.12</td>
<td>3.79 ± 0.19</td>
<td>4.30 ± 0.24</td>
<td>3.02 ± 0.04</td>
<td>3.11 ± 0.08</td>
<td>3.37 ± 0.10</td>
<td>3.69 ± 0.19</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>1.14 ± 0.04</td>
<td>1.94 ± 0.16</td>
<td>2.47 ± 0.24</td>
<td>3.03 ± 0.30</td>
<td>1.11 ± 0.04</td>
<td>1.42 ± 0.11</td>
<td>1.77 ± 0.15</td>
<td>2.16 ± 0.26</td>
</tr>
<tr>
<td>FS, %</td>
<td>62 ± 1</td>
<td>45 ± 3</td>
<td>38 ± 3</td>
<td>33 ± 3</td>
<td>63 ± 1</td>
<td>55 ± 2</td>
<td>49 ± 3</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>SEPth, mm</td>
<td>0.77 ± 0.02</td>
<td>1.07 ± 0.04</td>
<td>1.01 ± 0.04</td>
<td>0.98 ± 0.05</td>
<td>0.76 ± 0.02</td>
<td>1.12 ± 0.03</td>
<td>1.11 ± 0.04</td>
<td>1.06 ± 0.05</td>
</tr>
<tr>
<td>PWth, mm</td>
<td>0.73 ± 0.02</td>
<td>1.00 ± 0.03</td>
<td>1.01 ± 0.04</td>
<td>0.97 ± 0.06</td>
<td>0.74 ± 0.01</td>
<td>1.08 ± 0.03</td>
<td>1.10 ± 0.04</td>
<td>1.04 ± 0.05</td>
</tr>
<tr>
<td>Mean Vcf, m/s</td>
<td>4.28 ± 0.10</td>
<td>3.04 ± 0.20</td>
<td>2.50 ± 0.19</td>
<td>2.14 ± 0.19</td>
<td>4.36 ± 0.08</td>
<td>3.74 ± 0.16</td>
<td>3.32 ± 0.19</td>
<td>2.96 ± 0.24</td>
</tr>
<tr>
<td>BW, g</td>
<td>27.5 ± 1.2</td>
<td>26.6 ± 1.0</td>
<td>29.0 ± 1.0</td>
<td>28.9 ± 1.2</td>
<td>6.94 ± 0.30</td>
<td>5.70 ± 0.29</td>
<td>90.4 ± 6.3</td>
<td></td>
</tr>
<tr>
<td>LWV/BW, mg/g</td>
<td>71.5 ± 3.3</td>
<td>6.94 ± 0.30</td>
<td>5.70 ± 0.29</td>
<td>90.4 ± 6.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSPG, mmHg</td>
<td>71.5 ± 3.3</td>
<td>6.94 ± 0.30</td>
<td>5.70 ± 0.29</td>
<td>90.4 ± 6.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TSPG, trans-stenotic systolic pressure gradient as the difference between right carotid and left axillary artery systolic pressures. *P < 0.01, PI3Kγ-inact versus WT at same point; †P < 0.01, ‡P < 0.001 versus before TAC in same group. Two-way repeated measures ANOVA was used in the analysis of the data.
mice, wherein βARs were significantly reduced (Figure 7a). Since heart failure causes uncoupling of βARs from G proteins, we tested the ability for βARs to activate adenyl cyclase in pressure-overloaded WT and PI3Kγinact hearts. Importantly, there was complete preservation of adenyl cyclase activity in PI3Kγinact mice compared with the WT, despite the exposure to chronic pressure overload (Figure 7b). To determine whether the preservation of cyclase activity was the result of enhanced βAR–G protein coupling, competition binding was carried out on membrane fractions from the hearts of WT and PI3Kγinact banded mice. The number of βARs in the high-affinity state was significantly greater in PI3Kγinact hearts compared with WT hearts (P < 0.01) after 12 weeks of TAC and equal to the level in the WT sham (Figure 7c). These data show that βAR density and βAR–Gαs subunit of heterotrimeric G protein effector coupling was preserved in hearts of PI3Kγinact mice despite being subjected to chronic pressure overload, a stimulus that normally induces significant alterations in βAR signaling.

**PI3K and βAR function.** βAR downregulation and desensitization are hallmarks of heart failure and are believed to be secondary to the chronic activation of the sympathetic nervous system and increased catecholamines that occur with circulatory failure (2, 26). Recent data show that preservation of βAR function through βARK1 inhibition improves cardiac function in experimental models of heart failure (3–5). The mechanism for this benefit, however, is not entirely clear since βARK1 inhibition was carried out using overexpression of the βARK1-ct peptide (c-terminal region of βARK1) that also has the ability to sequester Gβγ subunits and as a result could inhibit activation of Gβγ-mediated signals. In this regard, our present study highlights a novel strategy to preserve βAR function under conditions of chronic βARK1 inhibition and desensitization with chronic catecholamine administration and ameliorates the development of cardiac dysfunction under conditions of chronic in vivo pressure overload. Our data show that overexpression of PI3Kγinact in the hearts of transgenic mice leads to a competitive displacement of all PI3K isoforms from the βARK1/PI3K complex such that translocation of βARK1 recruits catalytically inactive PI3K to activated receptors, leading to the inhibition of receptor internalization (Figure 7d). Intriguingly, we also show that the deletion of PI3Kγ in the heart is insufficient to prevent βAR dysfunction despite its positive effect on adenyl cyclase activity. These data suggest that the generation of PtdIns-3,4,5-P3 molecules localized within the activated receptor complex plays a critical role in regulating βAR recycling and preserving βAR function in vivo.

**Discussion.** In the present investigation we identify a unique role for PI3K in regulating the level and sensitivity of βAR function in the heart and suggest an important role for βARK dysfunction in pressure overload–induced heart failure. We show that cardiac-targeted overexpression of a catalytically inactive PI3K prevents βAR downregulation and desensitization with chronic catecholamine administration and ameliorates the development of cardiac dysfunction under conditions of chronic in vivo pressure overload.
liberated Gβγ subunits, thereby inhibiting βARK1-mediated phosphorylation of βARs (27). In contrast, overexpression of PI3Kγ\textsuperscript{inact} transgene does not inhibit receptor phosphorylation as we show by in vitro rhodopsin phosphorylation but, rather, prevents subsequent process involved in receptor internalization (8).

The effect on βAR function was found to be specific to the disruption of the βARK1/PI3K interaction, since mice that completely lack the PI3Kγ gene (PI3Kγ-KO) showed a similar downregulation of βARs and diminished agonist-stimulated adenyl cyclase activity when exposed to chronic catecholamines. This is particularly interesting given the recently identified role of PI3Kγ in regulating adenyl cyclase activity (9, 12). PI3Kγ-KO mice have been shown to have enhanced contractility and elevated basal and agonist-stimulated adenyl cyclase activity (ref. 9; Figure 4c). Despite this enhanced adenyl cyclase activity, however, chronic ISO administration leads to desensitization and downregulation of βARs. Importantly, βARK1-associated PI3K activity in the hearts of PI3Kγ-KO mice was similar compared with the WT, and we show that βARK1 interacts with the PI3Kγ isoform in the hearts of these mice (10). Based on the phenotype of these two mouse models (PI3Kγ\textsuperscript{inact} and PI3Kγ-KO), we postulate that overexpression of the dominant negative PI3K transgene leads to a reduction in βARK1-associated PI3K activity, which plays a critical role in preventing the downregulation and desensitization of the βARs under conditions of chronic agonist stimulation. Furthermore, the membrane recruitment of PI3K appears to be an early event in the regulation of βAR function and is consistent with our in vitro studies showing its role in receptor internalization within minutes of agonist stimulation (10). Thus, our data identify a new role for PI3K in maintaining the level of βARs in the heart. Whereas PI3Kγ may directly regulate cyclase activity (9), the recruitment of any active PI3K isoform to the receptor complex promotes the downregulation of βARs. Our data have important implications regarding the potential development of PI3Kγ inhibitors to augment cardiac contractility (9). Without preventing the βARK1-mediated recruitment of active PI3K to the cell membrane, a selective inhibitor of PI3Kγ would not prevent the βAR abnormalities or the contractility defects in heart failure.

Resensitization allows βARs to renew their ability to respond to ligand and has been shown to require internalization into intracellular compartments where the acidic environment allows for dephosphorylation of the receptor (28, 29). An interesting finding of this study is that resensitization of βARs in vivo may occur under conditions that prevent receptor internalization despite continuous exposure to agonist. One potential explanation for this phenomenon is the targeting of phosphatases to agonist-occupied receptors (30, 31), which could promote dephosphorylation of the receptor at the plasma membrane without the cycling into early endosomes. Alternatively, it is possible that βARs are internalized but undergo rapid recycling without being targeted for lysosomal degradation, a process thought to require PI3K activity (32, 33). Future studies that use in vitro models of βAR desensitization will be required to determine the exact mechanism of βAR resensitization in this system. Finally, we cannot exclude the possibility that the PI3Kγ\textsuperscript{inact} transgene affects some other signaling pathway that ameliorates cardiac dysfunction, leading to a reversal in the βAR defect.

PI3K and downstream cellular signaling. PI3Kγ\textsuperscript{inact} overexpression did not affect the immediate downstream signaling with either GPCR or growth factor stimulation and did not inhibit the development of cardiac hypertrophy to short-term pressure overload. Although basal pPKB levels are lower in the PI3Kγ\textsuperscript{inact} transgenic mice compared with the WT, acute and chronic stimulation leads to PKB activation possibly due to availability of liberated Gβγ to activate endogenous PI3Ks and/or through transactivation of tyrosine kinase receptors such as the epidermal growth factor (34, 35). This supports our understanding that PI3Kγ\textsuperscript{inact} overexpression specifically disrupts the βARK1/PI3K interaction without affecting the acute activation of receptor-mediated downstream PI3K signals. Increasing evidence supports a role for PI3K in determining cardiac growth (13). Cardiac overexpression of a catalytically inactive PI3Kα (dnPI3Kα) was shown to result in reduced heart weight and cardiomyocyte size compared with WT, whereas overexpression of constitutively active PI3Kα developed a hypertrophic phenotype suggesting a specific regulatory role for the PI3Kα isoform in the control of myocyte growth (13). Moreover, mice deficient in phosphatase and tensin homolog deleted on chromosome 10 (PTEN) showed spontaneous cardiac hypertrophy and reduced contractility, indicating a role of phosphatidylinositol signaling in cardiac growth (9). In contrast, in our study mice overexpressing PI3Kγ\textsuperscript{inact} had no effect on cardiac growth under normal or stressed conditions. The likely explanation for the difference in the mouse phenotypes relates to the different transgenes that were used in the two studies. We overexpressed the inactive PI3Kγ isoform that contained all the domains except for the ATP-binding site. In contrast, the dnPI3Kα transgene used by Shioli et al. (13) lacked the PIK domain that is necessary for its interaction with βARK1 (8). Thus, overexpression of dnPI3Kα would not displace endogenous PI3K from βARK1, but would act to sequester adaptor proteins involved in PI3Kα signaling pathways. Our data in this study show that competitive displacement of PI3K from the βARK1 complex is critical to preserve βAR function and prevent βAR downregulation in chronic disease states.

In conclusion, we demonstrate a new role for PI3K in regulating GPCR signaling in vivo. Overexpression of catalytically inactive PI3Kγ displaces active endogenous PI3K from βARK1 and leads to preservation of βAR function under conditions of chronic catecholamine administration and chronic pressure overload. Targeting PI3Kγ directly would be insufficient as a therapeutic treatment for heart failure, but additional studies are necessary to evaluate the potential for PI3Kγ as a therapeutic target in heart disease.
strategy, but, rather, one would need to disrupt recruitment of P13K to activated βARs to prevent receptor desensitization and downregulation. While the increased contractile phenotype of the P13Kγ-KO mice (9) suggests a direct action of P13Kγ on the catalytic activity of adenyl cyclase, displacement of endogenous active P13K from βARK1 is necessary to modulate receptor function. Finally, we show that normalization of βAR function is associated with the amelioration of the heart failure phenotype induced by pressure overload and suggests that βAR dysfunction may be involved in the pathogenesis of this disease. These findings may have important clinical implications for the treatment of heart failure, where circulating catecholamines are known to be increased leading to marked βAR dysfunction. Inhibition of βAR-localized P13K activity may therefore represent a novel therapeutic strategy to restore βAR function.

Acknowledgments
This work was supported in part by the NIH (grant HL-56687 to H.A. Rockman) and the Burroughs Wellcome Fund (to H.A. Rockman). J.J. Nienaber is a recipient of HL-56687 to H.A. Rockman) and the Burroughs Wellcome Fund and the National Heart, Lung, and Blood Institute (H.A. Rockman). We thank Weili Zou for her excellent technical assistance and Liza Barki-Harrington for her valuable critique and scientific insights.


