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G_i-Biased β_2AR Signaling Links GRK2 Upregulation to Heart Failure

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Abstract

Rationale—Phosphorylation of β_2-adrenergic receptor (β_2AR) by a family of serine/threonine kinases known as G protein-coupled receptor kinase (GRK) and protein kinase A (PKA) is a critical determinant of cardiac function. Upregulation of G protein-coupled receptor kinase 2 (GRK2) is a well-established causal factor of heart failure, but the underlying mechanism is poorly understood.

Objective—We seek to determine the relative contribution of PKA- and GRK-mediated phosphorylation of β_2AR to the receptor coupling to G_i signaling that attenuates cardiac reserve and contributes to the pathogenesis of heart failure in response to pressure overload.

Methods and Results—Overexpression of GRK2 led to a G_i-dependent decrease of contractile response to βAR stimulation in cultured mouse cardiomyocytes and *in vivo*. Importantly, cardiac-specific transgenic overexpression of a mutant β_2AR lacking PKA phosphorylation sites (PKA-TG), but not the wild type β_2AR (WT TG) or a mutant β_2AR lacking GRK sites (GRK- TG), led to exaggerated cardiac response to pressure overload, as manifested by markedly exacerbated cardiac maladaptive remodeling and failure, and early mortality. Furthermore, inhibition of G_i signaling with pertussis toxin restores cardiac function in heart failure associated with increased β_2AR to G_i coupling induced by removing PKA phosphorylation of the receptor and in GRK2

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transgenic mice, indicating that enhanced phosphorylation of β_2AR by GRK and resultant increase in G_i-biased β_2AR signaling play an important role in the development of heart failure.

Conclusions—Our data show that enhanced β_2AR phosphorylation by GRK, in addition to PKA, leads the receptor to G_i-biased signaling which, in turn, contributes to the pathogenesis of heart failure, marking G_i-biased β_2AR signaling as a primary event linking upregulation of GRK to cardiac maladaptive remodeling, failure and cardiodepression.

Keywords

β_2-adrenergic receptor; G protein-coupled receptor kinase; Heart failure; hypertrophy

Introduction

Despite major developments in both diagnosis and treatment, heart failure (HF) continues to be a leading cause of death and disability in western countries and will become the number one killer worldwide in 2020[1]. It has been controversial as to whether increased cAMP-PKA signaling is beneficial or detrimental in the context of heart failure. Patients with HF exhibit chronically enhanced PKA signaling,[2, 3] and transgenic mouse models with cardiac specific overexpression of β_1AR,[3] the a-subunit of G_s,[4] and the catalytic subunit of PKA[5] display HF phenotypes, suggesting overtly enhanced PKA signaling is cardiac detrimental. Over the past two decades, compelling evidence indicates that phosphorylation of βARs by another family of serine/threonine kinases known as GPCR kinases (GRKs) in the heart is a critical determinant of cardiac function and has been implicated in many pathological conditions including HF.[6] In humans or animal models with HF, chronic catecholamine elevation causes marked dysregulation of βARs, resulting in various molecular abnormalities, including upregulation of GRK2 and PTX-sensitive G_i proteins. Upregulation of both of these proteins have been implicated as causal factors in the development of HF. In particular, GRK2 is the most abundant and best-characterized GRK in the heart.[7] GRK2 expression and activity are markedly elevated and play a central role in the HF-associated defect in βAR signaling and cardiac dysfunction. Myocardial ischemia and hypertension in humans and animal models have also been associated with elevated GRK2 expression and activity.[6] These previous studies have defined GRK2 upregulation as an early common event in cardiac maladaptive remodeling and HF.

It has been shown that phosphorylation of β_2AR plays a crucial role in regulating differential G protein coupling of the receptor. Specifically, βb_2AR phosphorylation by PKA mediates the switch of coupling from G_s to G_i.[8, 9] Targeted transgenesis reveals discrete attenuator functions of GRK and PKA in airway β_2AR physiologic signaling.[10] Further studies have demonstrated that β_2AR coupling to G_i may be also dependent on the receptor internalization and recycling.[11–13] However, it is unclear whether GRK-mediated phosphorylation of β_2AR is involved in the regulation of β_2AR-coupled G_i signaling in heart. Because both GRK2 and G_i proteins are significantly elevated in HF caused by a multitude of etiologies,[14–19] we hypothesize that the well-documented detrimental effects of GRK2 in the failing heart may causatively link to enhanced β_2AR-coupled G_i signaling.

In the present study, we explored the mechanism which links pathological upregulation of GRK2 to the development of HF. We found both *in vivo* and *in vitro* that enhanced β_2AR phosphorylation by GRK2 leads the receptor to G_i-biased signaling, and that inhibition of the G_i signaling blocks heart failure in transgenic mice with cardiac-specific overexpression of GRK2 (GRK2-TG) or of a β_2AR mutant lacking all of the PKA phosphorylation sites (PKA- TG) subjected to pressure overload, marking G_i-biased β_2AR signaling as a primary event linking upregulation of GRK2 to cardiac maladaptive remodeling and failure.

Methods and Materials

Generation of Transgenic Mice

Flag-tagged human β_2AR mutants lacking either the putative GRK phosphorylation sites (GRK-) or the putative PKA phosphorylation sites (PKA-) (Fig. 2A&B) were subcloned into a pBluescript-based transgenic vector downstream of α-myosin heavy chain (α-MHC) gene promoter and upstream of the SV40 polyadenylation site. The detail sequences of the β_2AR PKA- and GRK- mutants were presented in Fig. 2B. Transgenic mice with cardiac-specific overexpression of wild type human β_2AR (WT TG) were imported from Dr. Gerald Dorn Lab.

Animal models

We used male non-transgenic mice (**NTG**), transgenic mice with cardiac-specific overexpression of wild type human β_2AR (**WT TG**), or PKA-phosphodeficient β_2AR (**PKA- TG**), or GRK-phosphodeficient β_2AR (**GRK- TG**), and their littermate controls at 12–16 weeks of age. In addition, male transgenic mice with cardiac-specific overexpression of GRK2 (**GRK2 TG**) and their littermate control mice (**LC**) were used in a subset of experiments. Pressure overload was produced by transverse aortic constriction (TAC) as previously described [20].

Supplemental Materials on Detailed Methods

See the online supplemental materials for detailed methods regarding *in vivo* assessment of mouse cardiac contractility by echocardiography (ECHO) and Millar system, radioligand binding assay, western blot analysis, adult mouse cardiac myocyte culture and adenoviral gene transfer, cardiomyocyte contraction measurements, histological analysis, cAMP assay, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and statistical analysis.

Results

Overexpression of GRK2 Causes βAR Dysfunction by Enhancing G_i Signaling in Cultured Cardiomyocytes And in Vivo

We have recently demonstrated that G_i-biased β_2AR signaling is dependent on agonist stimulation, and that prolonged absence of agonist stimulation leads to uncoupling of β_2AR from the G_i signaling, as is the case in adult mouse cardiomyocytes cultured for 24 hours.[21] Consistent with the previous notion, in adult mouse cardiomyocytes cultured and infected by Adv-β-Gal for 24 hours, β_2AR stimulation with zinterol led to a full contractile response which was insensitive to pertussis toxin (PTX) treatment (Figure 1 A&B). Importantly, overexpression of GRK2 with adenoviral gene transfer suppressed β_2AR-mediated contractile response and the inhibitory effect of GRK2 was fully abolished by disrupting G_i signaling with PTX (Figure 1A & B). Prolonged stimulation of cardiomyocytes with isoproterenol (ISO, 1 nM) in the presence or absence of PTX did not change the expression of GRK2 (Supplemental material, online Figure I). These results demonstrate, for the first time, that overexpression of GRK2 enhances G_i-biased β_2AR signaling. To further determine whether *in vivo* overexpression of GRK2 in the heart can facilitate β_2AR-couled G_i signaling and, if so, whether the enhanced G_i signaling is involved in GRK2-mediated βAR dysfunction, we took advantage of transgenic mice with cardiac-specific overexpression of GRK2.[22] Consistent with our previous studies on these mice,[22] *in vivo* experiments revealed that βAR-induced increases in cardiac contractility and relaxation, as measured by left ventricular (LV) $+dP/dt_{max}$ and $-dP/dt_{min}$, respectively, were markedly suppressed in mice overexpressing GRK2 (GRK2 TG mice) compared to wild type littermate controls (LC mice) (Figure 1C&D). Remarkably, disruption of G_i signaling with

PTX fully restored cardiac contractile response to βAR stimulation with ISO in GRK2 TG mice without altering the effect of ISO in LC mice (Figure 1C&D), indicating that overexpression of GRK2-induced cardiac βAR dysfunction is mediated by enhanced G_i signaling.

Augmentation of GRK-mediated β_2AR Phosphorylation Leads To Exacerbated Cardiac Hypertrophy, Heart Failure, and Early Mortality in Response to Pressure Overload

Since β_2AR can be phosphorylated by at least two types of protein kinases, PKA and GRK (Figure 2A), we sought to distinguish the potential role of GRK- versus PKA-mediated β_2AR phosphorylation in the pathogenesis of HF *in vivo*. The experiments took advantage of three different transgenic mouse models we developed: mice cardiac-specifically overexpressed the WT human β_2AR (WT TG) or β_2AR mutants in which all of the PKA phosphorylation sites or all of the GRK phosphorylation sites were substituted by alanine (A) or glycine (G) (namely PKA-TG or GRK2- TG, respectively) (Figure 2 A&B) at a matched receptor density (Figure 2C). The receptor densities in hearts from WT TG, PKA-TG, and GRK- TG mice were detected by radioligand binding assay (856±45, 828±33, and 850±23 fmole/mg protein, n=3–4, respectively) (Figure 2C). As compared to non-transgenic (NTG) mice, overexpression of the WT β_2AR led to profoundly increased phosphorylation of β_2AR at both GRK and PKA sites assayed by site-specific antibodies (Figure 2D). As expected, PKA- TG mice showed a clear increase in phosphorylation of β_2AR by GRK but not PKA, whereas GRK- TG mice exhibited augmented phosphorylation of β_2AR by PKA but not GRK (Figure 2D). Thus, GRK- and PKA-mediated phosphorylation of β_2AR was selectively enhanced in PKA- TG and GRK- TG mice, respectively.

To investigate whether β_2AR phosphorylation by GRK and PKA is differentially involved in the regulation of cardiac structure and function, NTG or PKA- TG, GRK- TG, or WT TG mice were subjected to pressure overload by transverse aortic constriction (TAC). There was no obvious phenotypic difference in cardiac anatomy and function among the genotypes under resting conditions at 4–5 months old of age, as assessed by echocardiography (ECHO) (Figure 3A&B). However, after TAC for 5 weeks, PKA- TG mice developed severe left ventricular dilation and contractile dysfunction (Figure 3 A&B) and increased mortality rate (Figure 3C). Cardiac dysfunction occurred as early as one week after TAC and progressively worse as TAC continued only in PKA- TG mice (Figure 3B). In contrast, GRK- TG mice were not different from NTG or WT TG mice in terms of cardiac anatomy and function in response to TAC. Furthermore, PKA- TG mice displayed markedly exaggerated cardiac hypertrophic response to TAC, as manifested by significantly increased heart size (Figure 4A), heart/body ratio (Figure 4B), and cardiomycoyte size (supplemental material, online Figure II). It is also important to note that PKA- TG mice, but not NTG or WT TG or GRK-TG, showed increased fibrosis after TAC for 5 weeks (Figure 4C&D). Thus, pressure overload led to more severe ventricular dilation with increased end-diastolic and end-systolic dimensions, reduced fractional shortening (FS) (Figure 3), and exacerbated cardiac maladaptive remodeling (Figure 4) in PKA- TG mice but not in other groups including NTG, WT TG and GRK- TG mice.

Accelerated Heart Failure of PKA- TG Mice Is Associated With Enhanced β_2AR-coupled G_i Signaling

More detailed examination of cardiac function was carried out by invasive pressure-volume analysis. Pressure-volume loops were measured before and during transient reduction of chamber preload to generate specific end-systolic (ESPVR) and end-diastolic (EDPVR) pressure volume relations (Figure 5). After TAC for 5 weeks, NTG hearts had enhanced effective arterial elastance (Ea; an index of total ventricular afterload) and slope of end-systolic pressure-volume relation with reduced ejection fraction (EF), characteristics of

hypertrophy induced by sustained pressure overload. Under resting conditions, PKA- TG, GRK- TG, and WT TG mice had similar increases in LV peak systolic pressure and basal systolic function with a slight upward and leftward shift of their ESPV compared to NTG mice. TAC for 5 weeks triggered distinct phenotypes between PKA- TG mice and GRK- TG or WT TG mice in terms of the progression of cardiac dysfunction. As shown by the representative examples, in the GRK-TG and WT TG mice, enhancements in resting Ea and slopes of ESPVR (before TAC) were slightly increased after 5 weeks of TAC, consistent with a functional response with feature of cardiac hypertrophy (Figure 5A). This did not occur in PKA- TG mice. Notably, PKA- TG mice displayed ventricular dilation, suppressed +dP/dt, reduced Ea, decreased EF associated with a rightward shift of pressure-volume relation, and a decrease in the slope of end systolic pressure-volume relation (Figure 5A&B). After 5 weeks of TAC, PKA- mice demonstrated a more significant decrease in maximal rate of pressure decline (−dP/dt), although a relaxation index tau was comparable in GRK- TG, and PKA- TG mice (Figure 5C).

The aforementioned data clearly indicate that PKA- TG mice are more vulnerable to pressure overload, suggesting that selectively enhancing β_2AR phosphorylation by GRK, but not PKA, profoundly exacerbates pressure overload-induced cardiac maladaptive remodeling and dysfunction. Next, we sought to decipher the mechanism underlying the distinct phenotypes of PKA- TG mice versus those of GRK- TG mice. Western blotting revealed that the increases in GRK2 and G_i protein abundance were significantly greater in hearts from PKA-TG mice relative to hearts from NTG or WT TG or GRK- TG mice, although both GRK2 and G_i proteins were markedly elevated in all genotypes after 5 weeks TAC (Figure 6A&B). Concomitantly, hearts from PKA- TG mice failed to compensate pressure overload even one week after TAC, resulting in significantly diminished cardiac contractility in PKA- TG mice compared to that in other genotypes (NTG or WT TG or GRK2- TG) (Figure 6C). Importantly, disruption of the G_i signaling with PTX treatment minimized the difference between PKA- TG mice and other groups (Figure 6C). In fact, in an early stage of heart failure (one week after TAC), PTX treatment fully restored cardiac function in PKA- TG mice, while in the later stage of heart failure (5 weeks after TAC), PTX substantially improved cardiac function in PKA- TG mice (Figure 6C), highlighting that enhanced GRK2 and subsequent enhancement of G_i-biased β_2AR signaling play a crucial role in the triggering and worsening TAC-induced cardiac maladaptive remodeling and failure.

In principle, the cardiac dysfunction in PKA- TG mice could be due to an enhancement in the receptor to G_i signaling for attenuation of tonic cAMP signaling or due to cardiac adaptive remodeling for structure changes in myocardium or both alterations. To further investigate this issue, we measured basal and ISO-induced cAMP formation in cardiomyocytes from NTG or TG mice expressing WT or mutant β_2AR. The present data demonstrated that spontaneous β_2AR activity is increased in all of the transgenic mice expressing WT or mutant β_2AR, as evidenced by their increased cAMP baselines (Figure 6D and supplementary data, online Figure III), consistent with the hemodynamic data (Figure 5). It is noteworthy that the decay of ISO-induced cAMP accumulation declines faster in cells from PKA- TG mice than that in cells from WT TG or GRK- TG mice. Since the decay of ISO-induced cAMP accumulation is sensitive to PTX, our data indicates that β_2AR/G_i coupling is enhanced in PKA- TG mice. Thus, the cardiac dysfunction of PKA- TG mice is likely due to enhanced G_i signaling, which, in turn, contributes to cardiac adaptive remodeling and the progression of heart failure.

Discussion

The present study has revealed three major findings. First, phosphorylation of β_2AR by GRK as well as PKA is a primary determinant of the receptor-coupled G_i signaling. Second, overexpression of GRK2 enhances G_i-biased β_2AR signaling, which subsequently negates cardiac contractile response to βAR stimulation in a PTX-sensitive manner in GRK2 transgenic mice and cultured mouse cardiomyocytes. Third, selective augmentation of GRK-mediated β_2AR phosphorylation in PKA- TG mice renders the heart more vulnerable to pressure overload and inhibition of G_i signaling can restore cardiac function of PKA- TG mice. In contrast, a selective increase in PKA-mediated phosphorylation of β_2AR does not affect cardiac response to pressure overload in GRK- TG mice compared to NTG or WT TG mice. Therefore, we conclude that cardiac detrimental effects of GRK2 upregulation are mediated, at least in part, by enhanced β_2AR-coupled G_i signaling which, in turn, contributes to the pathogenesis and progression of HF. Thus, both GRK2 and β_2AR-coupled G_i signaling may offer novel therapeutic opportunities for the treatment of heart failure.

Role of GRK2 in Normal and Failing Hearts

In our previous studies, we have shown that cardiac-specific overexpression of GRK2 to the levels seen in human HF suppresses cardiac contractile response to βAR stimulation with ISO,[22] indicating that myocardial overexpression of GRK2 triggers βAR desensitization *in vivo*. In contrast, overexpression of the peptide inhibitor of GRK2, GRK2-ct, enhances cardiac contractility and relaxation.[22] Thus, GRK2 expression level has an important impact on cardiac performance under normal conditions. This perception has been further elucidated in recent studies on GRK2 knockout mouse models.[32]

In the failing heart, adrenergic overdriving occurs early in the progression to HF, as evidenced by increased catecholamine levels before the onset of HF.[33] As a result, the expression and activity of GRK2 are elevated in the failing heart. Previous studies have shown upregulation of GRK2 is essentially involved in the pathogenesis of HF by further diminishing adrenergic responsiveness. Indeed, two recent clinical studies indicate that lymphocyte GRK2 decreases with angiotensin-converting enzyme (ACE) inhibitor therapy in class II HF,[34] and that lymphocyte and myocardial GRK2 levels decrease, while bAR responsiveness increases, after mechanical left ventricular support in end-stage HF.[26] Using heterozygous GRK2 knockouts with 50% diminished GRK2 expression, recent studies have established a dose-response between GRK2 levels and suppression of cardiac contractile function in HF.[27, 28] Indeed, in various experimental models of HF, inhibition of GRK2 with GRK2-ct improves cardiac function by restoring βAR signaling (for review see refs [6, 35]). Thus, upregulation of GRK2 and resultant βAR desensitization are initially adaptive compensation of the heart, but when exaggerated, causes cardiac maladaptive remodeling and HF.

The beneficial effect of restoration of βAR signaling through inhibiting GRK2 activity appears to contradict clinical convention that -blockade is widely used to treat patients with HF and chronic β-agonist (i.e., catecholamine) stimulation leads to deleterious effects. However, a close inspection reveals that the detrimental effects of catecholamines are mainly mediated by stimulation of β_1ARs, which triggers myocyte apoptosis and arrhythmogenic events. In contrast, the present study has shown that inhibition of GRK2-mediated β_2AR-coupled G_i signaling can normalize cardiac contractile response and ameliorate maladaptive remodeling. In this regard, our recent *in vivo* studies on a rat ischemic HF model have demonstrated that selective activation of β_2AR can, indeed, improve cardiac function and reduce maladaptive remodeling as well as arrhythmogenic events.[36] Taken together, we propose that a combination of GRK2 inhibition and β_2AR

activation with β_1-blocker therapy may provide a more effective therapy for the treatment of HF.

Role of GRK-mediated β_2AR Phosphorylation in Promoting G_i-biased signaling and Its Implications in Heart Failure

While βAR is classically viewed as a prototypical G_s-coupled receptor, compelling evidence indicates that β_2AR couples dually to G_s and G_i proteins.[37] We and others have previously shown that G_i-biased β_2AR signaling may protect cardiomyocytes against various insulting stimuli induced apoptosis.[38, 39] In addition, it has been shown that inhibition of G_i signaling worsens cardiac outcomes in response to ischemia/reperfusion injury and myocardial infarction,[40] and that GRK inhibition with the peptide inhibitor, GRK2-ct, is cardioprotective, at least in part, due to increasing G_i-biased β_2AR signaling.[41] Furthermore, recent studies have shown that ablation of β_2AR, indeed, worsens pressure overload-induced cardiac hypertrophy and remodeling in mice.[42] These previous studies seem to contradictory to the present conclusion that an augmentation in GRK-mediated β_2AR-G_i signaling contributes to cardiac maladaptive remodeling and failure under TAC in PKA- TG mice. To address this question, we have examined the potential effect of TAC on myocardial cell apoptosis in all four groups of mice (NTG, WT-TG, GRK-TG, PKA-TG) and found no genotype-related difference in TAC-triggered myocardial apoptosis (supplemental material, online Figure IV). In addition, we have shown that PTX enhances ISO-induced cardiomyocyte death, whereas β_2AR stimulation with zinterol protects cardiomyocytes in PTX-sensitive manner (supplemental material, online Figure V), consistent with the previous notion that β_2AR-G_i signaling is cardiac protective. However, cardiomyocytes from PKA- TG mice are more vulnerable than those from other groups (supplementary data, online Figure V), although β_2AR-G_i signaling is markedly enhanced in PKA- TG mice. Thus, it merits future investigation to elucidate the exact mechanism underlying cardiac maladaptive remodeling and failure associated with enhanced GRK-mediated β_2AR/G_i signaling in PKA- TG mice under TAC.

During prolonged agonist stimulation, GRK2 plays a predominant role in desensitizing βARs and has been implicated as a causal factor of HF, but the underlying etiological mechanism is unknown until now. Traditionally, it has been shown that GRK-mediated phosphorylation of βAR inhibits the interaction between activated receptor and G proteins via recruiting β-arrestins, which bind to phosphorylated βAR and sterically block the receptor coupling to the β subunit of G_s protein.[43] In this study, however, we have provided multiple lines of evidence to show that increased phosphorylation of β_2AR by GRK facilitates the receptor to G_i signaling which, in turn, results in cardiac contractile dysfunction and maladaptive remodeling. The present findings have also unraveled a novel function of the prototypical GRK, GRK2, in switching G_s- to G_i-biased β_2AR signaling. Thus, in addition to PKA, GRK plays an important role in sorting β_2AR to G_i-biased signaling pathway in response to enhanced catecholamine stimulation, as is the case in the failing heart.

It is noteworthy that, while the β_1AR subtype does not couple to G_i under normal conditions, β_1AR-mediated contractile response is cross-inhibited by enhanced G_i-biased β_2AR signaling in the failing heart.[29, 44] The reinforced GRK-dependent G_i-biased β_2AR signaling is likely also responsible for GRK2 overexpression-induced dysfunction of β_1AR in addition to the defect of β_2AR contractile response in these transgenic mice. This idea is, indeed, corroborated by the fact that disruption of G_i signaling fully, rather than partially, restores the nonselective βAR agonist, ISO, induced positive inotropic effect in transgenic mice overexpressing cardiac GRK2 (Fig. 1C&D). Thus, similar to the situation of the failing heart,[14, 44, 45] enhanced β_2AR-coupled G_i signaling is responsible for the defects of both β_1AR and β_2AR signaling in the GRK2 TG mice, and that the previously reported beneficial

effects of GRK2-ct in improving the function of the failing heart [23] is mediated, at least in part, by attenuating GRK-dependent G_i-biasedβ_2AR signaling.

It is known that overexpression of βARs may alter their signaling behavior and G protein coupling properties.46 Overexpression of the PKA-phosphodeficient β_2AR mutant in cells and in transgenic mice may complicate our conclusion on the functional consequence of GRK-mediated phosphorylation of β_2AR in facilitating G_i-biased β_2AR signaling because of possible disruptions of signaling events mediated by other molecules such as G_s, β-arrestins, and receptor trafficking,[13] although the present study has used WT TG and GRK2- TG for comparison. This technical limitation should be taken into consideration, when the present data is interpreted.

In summary, we have revealed a novel function of GRK in promoting G_i-biased β_2AR signaling that compromises cardiac reserve and contributes to the pathogenesis of HF. In the failing heart, enhanced expression and activity of GRK2 and G_i proteins further promote G_i-biased β_2AR signaling, thus negating β_1AR- and β_2AR-mediated cardiac reserve function, resulting in cardiac maladaptive remodeling and failure. These *in vitro* and *in vivo* results have not only revealed a fundamental function and new mechanism of action of GRK2, the best characterized GRKs, in facilitating G_i-biased β_2AR signaling, but also defined the β_2AR-G_i signaling as an essential link between pathologic upregulation of GRK and the development of heart failure. As a well-established pathogenic factor of heart failure, GRK2 and resultant G_i-biased β_2AR signaling may present important therapeutic targets for the treatment of heart failure caused by various etiologies.

Novelty and Significance

What is Known?

- Phosphorylation of G-protein coupled receptor alters the selectivity of the receptor for G protein coupling.
- β_2-adrenergic receptor (β_2-AR) phosphorylation by protein kinase A (PKA) is a critical determinant in its signaling to distinct G proteins such as G_s versus G_i.
- β_2-AR mediated G_i signaling elicits cardiac protective effects.
- Upregulation of G protein-coupled receptor kinase (GRK) is a well-established causal factor of heart failure.

What New Information Does This Article Contribute?

- Overexpression of GRK2 profoundly enhances G_i-biased β_2AR G_i signaling *in vivo* and *in vitro*, whereas inhibition of GRK2 markedly suppresses the β_2AR-coupled G_i signaling.
- Phosphorylation of β_2AR by GRK as well as PKA is an important determinant for the G_i-biased β_2AR signaling.
- In transgenic mice with cardiac-specific overexpression of a β_2AR mutant lacking PKA phosphorylation sites (PKA- TG), augmentation of β_2AR phosphorylation by GRK leads to exaggerated cardiac response to pressure overload, resulting in increased G_i-biased β_2AR, markedly exacerbated cardiac maladaptive remodeling and failure, leading to early mortality.
- In contrast, enhancement of PKA-mediated phosphorylation of β_2AR in mice overexpressing a β_2AR mutant lacking GRK phosphorylation sites (GRK-TG) does not alter cardiac anatomy or function when compared with non-

transgenic mice (NTG) or transgenic mice overexpressing the wild type β_2AR (WT TG) at a matched receptor density, indicating that phosphorylation of β_2AR by GRK, but not by PKA, contributes to the pathogenesis of heart failure.

- Inhibition of G_i signaling with pertussis toxin restores cardiac function in PKA- TG mice with pressure overload-induced heart failure and in GRK transgenic mice.
- Enhanced β_2AR coupling to G_i signaling may induce cardiac protective or detrimental effects.

Our *in vitro* and *in vivo* results reveal a fundamental function and new mechanism of action of GRK in facilitating G_i-biased β_2AR signaling by phosphorylation of β_2AR. We found that define β_2AR-G_i signaling is an essential linker between pathologic upregulation of GRK and the development of heart failure. As a well-established pathogenic factor of heart failure, GRK and resultant G_i-biased β_2AR signaling may be important therapeutic targets for the treatment of heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations and Acronyms

Adv	adenovirus
GPCR	G protein-coupled receptor
β-AR	β-adrenergic receptor
ISO	isoproterenol
FBS	fetal bovine serum
CGP	CGP20712A
ICI	ICI 118,551
MEM	minimal essential medium
[^{125}I]-ICYP	[^{125}I]-iodocyanopindolol
m.o.i.	multiplicity of infection
PKA	protein kinase A
PBS	phosphate buffered saline
PKI	protein Kinase inhibitor
PTX	pertussis toxin
NTG	non-transgenic mice
WT TG	transgenic mice overexpressing wild type human β_2-AR
GRK- TG	transgenic mice expressing a β_2AR mutant lacking GRK phosphorylation sites
PKA- TG	transgenic mice expressing a β_2AR mutant lacking PKA phosphorylation sites

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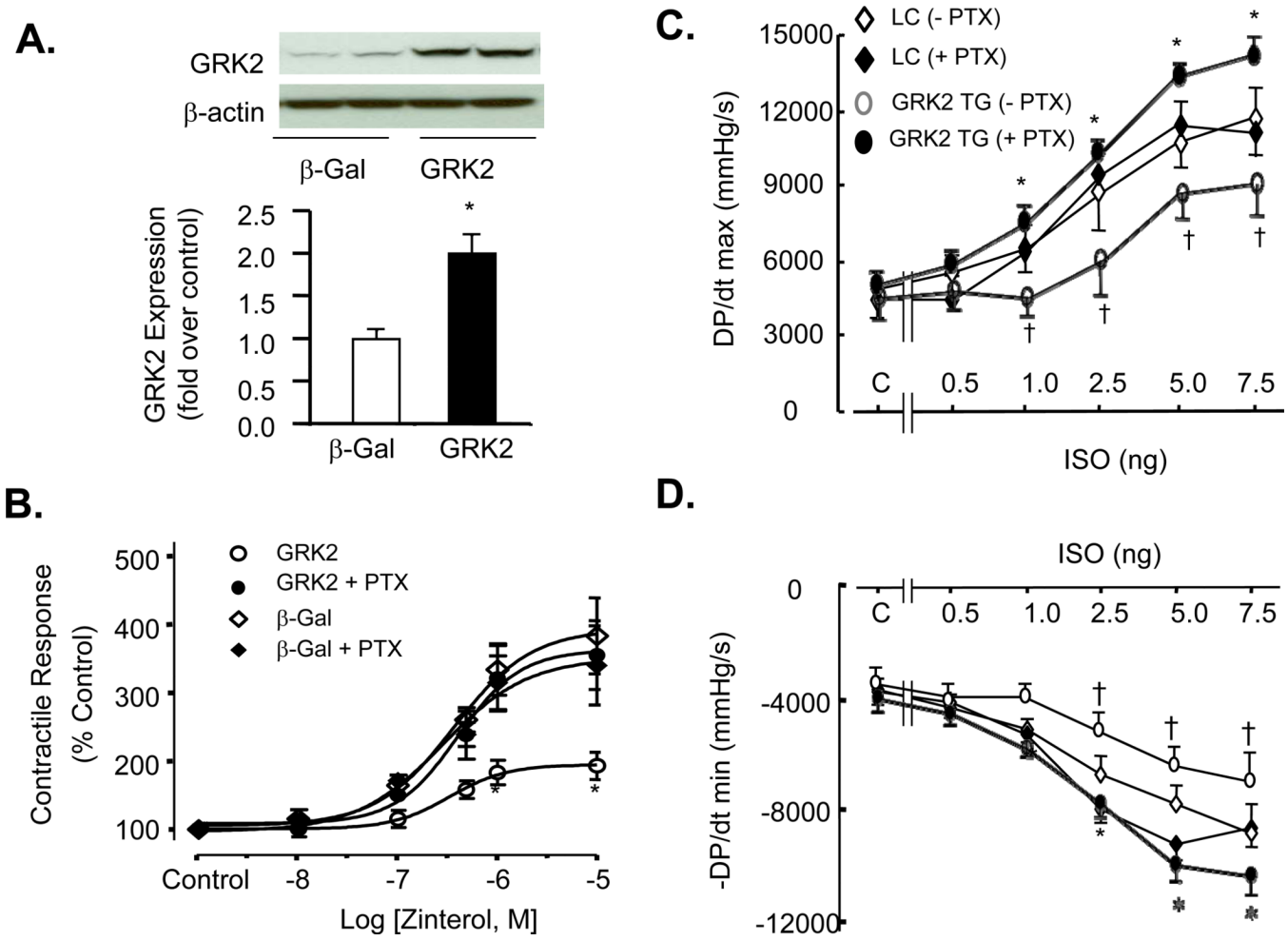


Figure 1. Inhibition of G_i by pertussis toxin (PTX) enhances βAR-mediated contractile response in cultured adult mouse cardiomyocytes overexpressing GRK2 and in GRK2 transgenic mice (GRK2 TG) in vivo

(A) A representative Western blot (top panel) of GRK2 in cultured adult mouse cardiomyocytes infected with adenovirus expressing GRK2 (Adv-GRK2) or β-Gal (Adv-β-Gal) at m.o.i. 100 for 24 h and quantified data (bottom panel) (n=4; *p<0.01 *v.s.* β-Gal). **(B)** Increased GRK2 expression enhances β_2AR to G_i signaling in adult mouse cardiomyocytes cultured for 24 h. In cultured cardiomyocytes infected with Adv-GRK2 or Adv-β-Gal (m.o.i. 100), overexpression of GRK2 enhances the receptor to G_i signaling, as evidenced by PTX-induced augmentation of β_2AR contractile response. *P<0.001 *v.s.* other three groups with a two-way repeated measures analysis of variance (ANOVA); **(C, D)** Disruption of G_i signaling with PTX abolishes GRK2-induced dysfunction of βAR contractile response in GRK2 TG mice. In vivo assessment of left ventricular (LV) function of GRK2 TG mice and littermate control (LC) mice (n=6–7 in each group; *P<0.001 *v.s.* GRK2 TG mice without PTX with a two-way repeated measures analysis of variance (ANOVA); †p<0.05 GRK2 TG *v.s.* LC in the absence of PTX with post hoc testing and Bonferroni's F test). For single cell experiments (panel B), cells were pretreated with PTX (1.5 μg/ml) 3 hours before agonist stimulation. For in vivo studies in panels C&D, one dose of PTX was administered i.p. 72 hours before hemodynamic measurements.

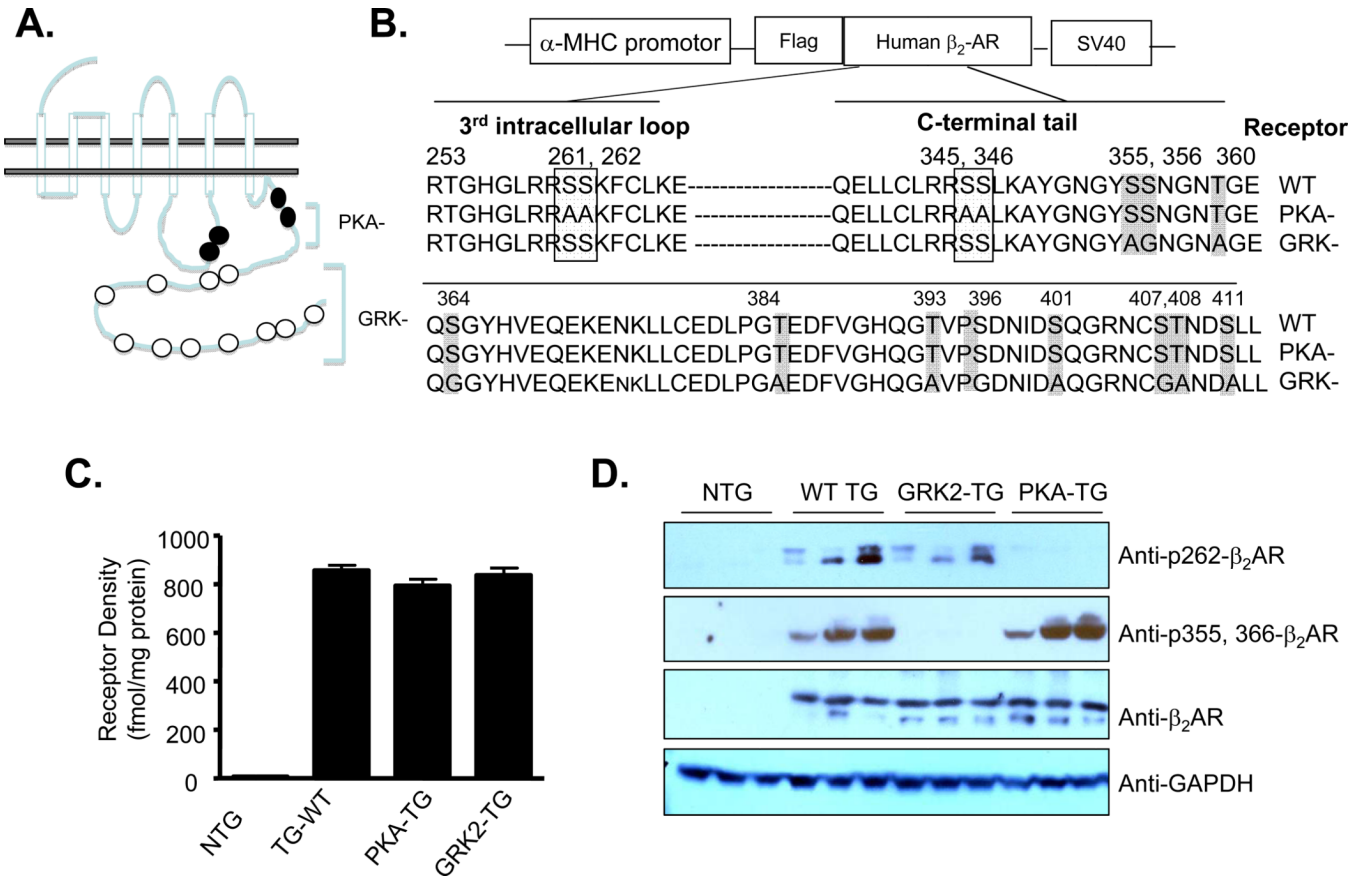


Figure 2. Transgenic mice with cardiac-specific overexpression of wild type (WT) human β_2AR or its mutants lacking PKA or GRK phosphorylation sites

(A) Schematic presentation showing β_2AR phosphorylation sites for PKA or GRK. **(B)** The specific sequences of WT β_2AR or its mutants lacking PKA (PKA-) or GRK (GRK-) phosphorylation sites. **(C)** The β_2AR density was 9.6 ± 1.3 fmol/ mg protein and total βAR density was 28.6 ± 3.4 fmol/ mg protein in NTG mice (n=6). In transgenic mice, β_2AR density was 856 ± 45, 828 ± 33 and 850 ± 23 fmol/mg protein for WT TG, PKA- TG, and GRK- TG mice, respectively, (n=6 for each group). **(D)** Phosphorylation of β_2AR in PKA or GRK sites were assayed by Western blot using a site-specific antibody reacting with phosphorylated β_2AR at a PKA site (aa262) or GRK sites (aa355 and aa366). The antibodies were raised against the peptides CDRTGHGLRRSpSKF-NH2 for the anti-pSer262 PKA site (clone 2G3) and CKAYGNGYpSpSNGN-NH2 for the anti-pS (Ser355, 356) (clone 5C3). Total expression of β_2AR in transgenic mouse hearts was detected by Western blot using an antibody reacting with β_2ARs.

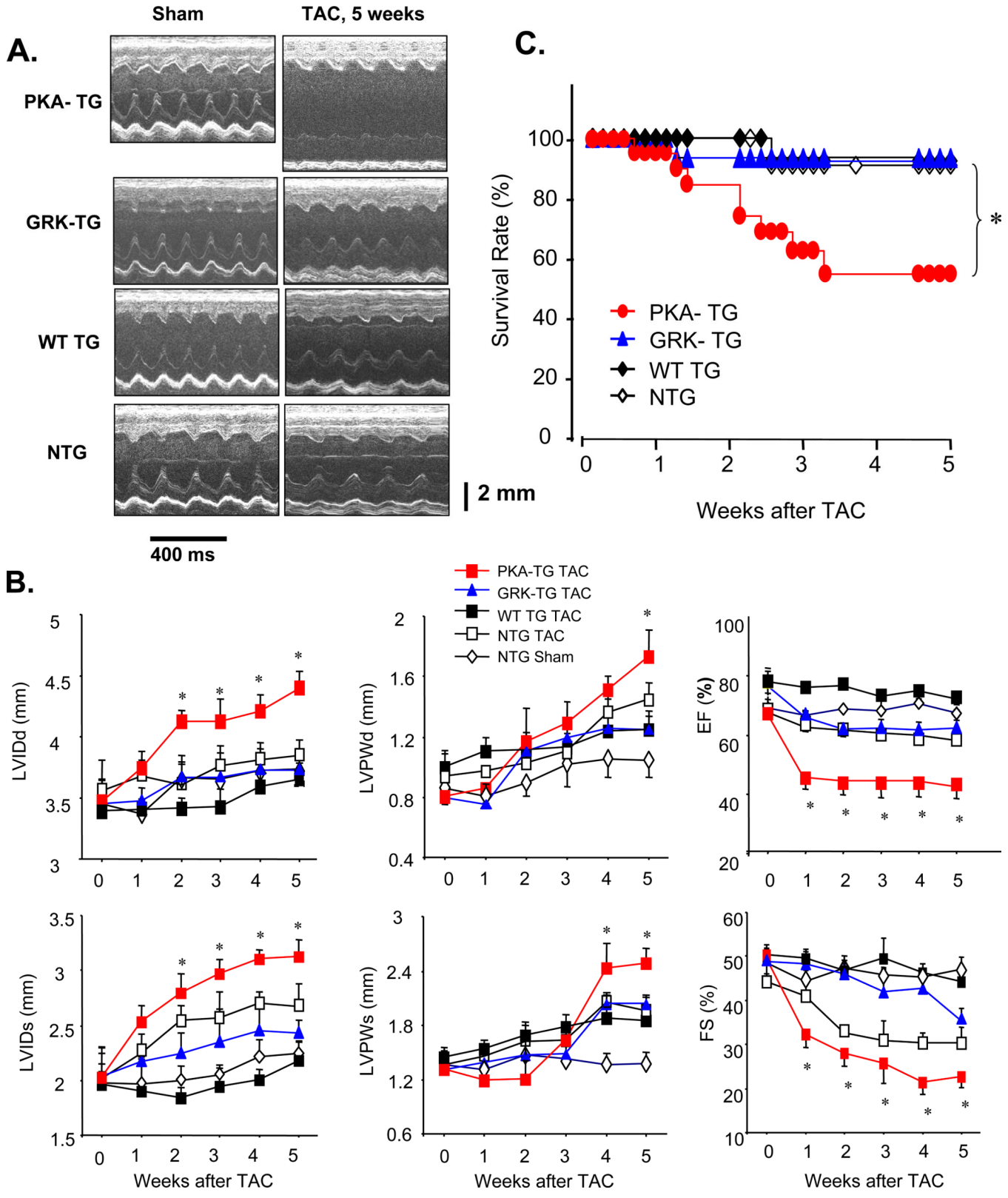


Figure 3. Overexpression of β_2AR mutant lacking PKA phosphorylation sites increases adverse pressure overload-induced ventricular remodeling

(A) Representative serial M-mode echocardiography in conscious NTG, WT TG, PKA- TG or GRK2- TG mice measured before and 5 weeks after TAC. **(B)** Summary data of Left Ventricular Internal Dimension-Diastole (LVIDd), Left Ventricular Internal Dimension-Systole (LVIDs), Left Ventricular Posterior Wall Dimensions-diastole (LVPWd), Left Ventricular Posterior Wall Dimensions-Systole (LVPWDs), Ejection Fraction (EF), and Fractional Shorting (FS). Data are presented as means ± SE (n = 8–12 in each group) and data was analyzed with a two-way repeated measures analysis of variance (ANOVA). Bonferroni correction was applied for multiple comparisons. *p<0.05 PKA- TG *v.s.* other four groups. **(C)** Survival curves. Kaplan-Meier Survival Analysis, *P<0.05 PKA- TG v.s. other three groups (GRK2- TG, WT TG, and NTG) (n=28, 25, 24 and 25 for PKA- TG, WT TG, GRK2- TG, and NTG, respectively).

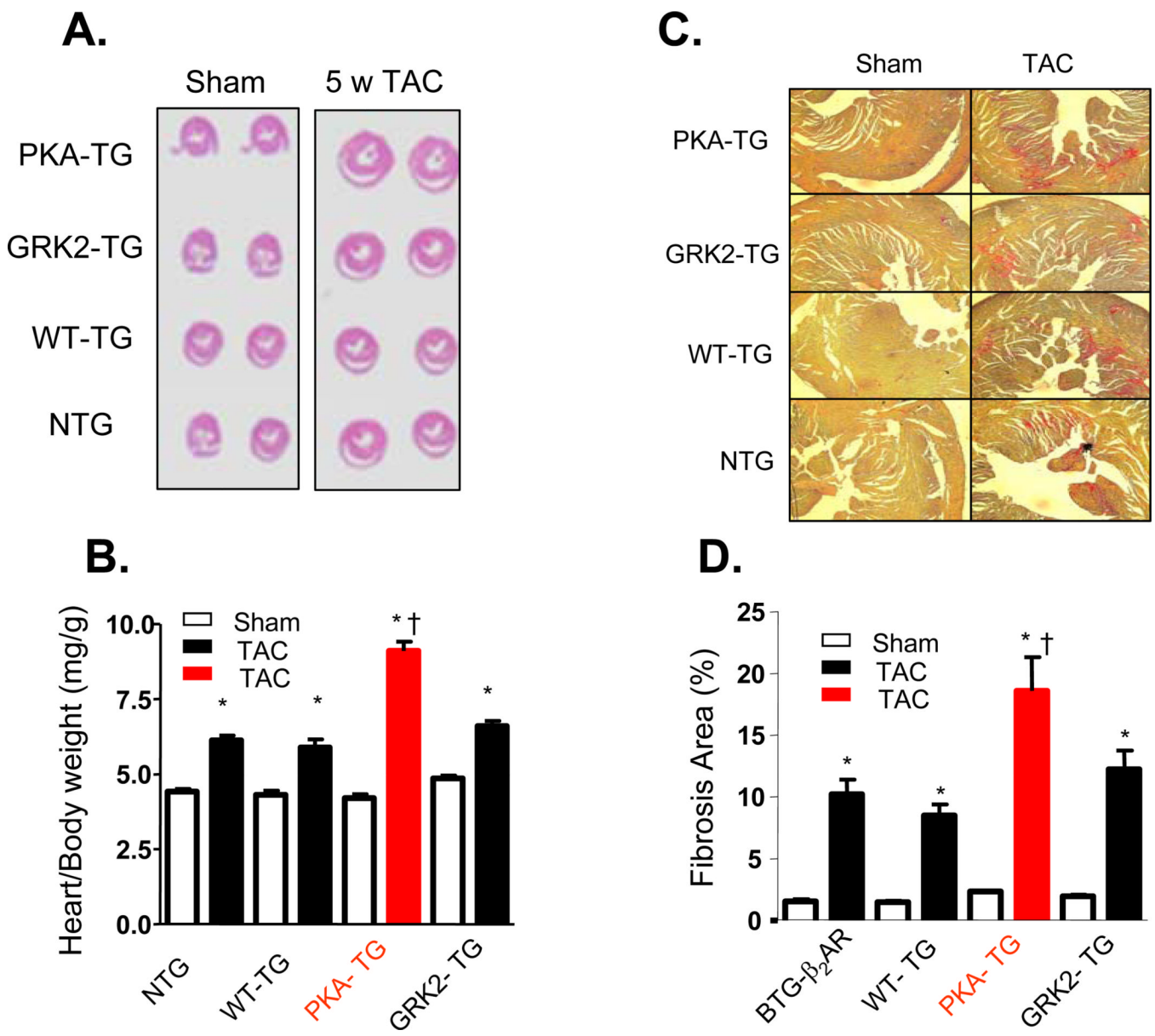
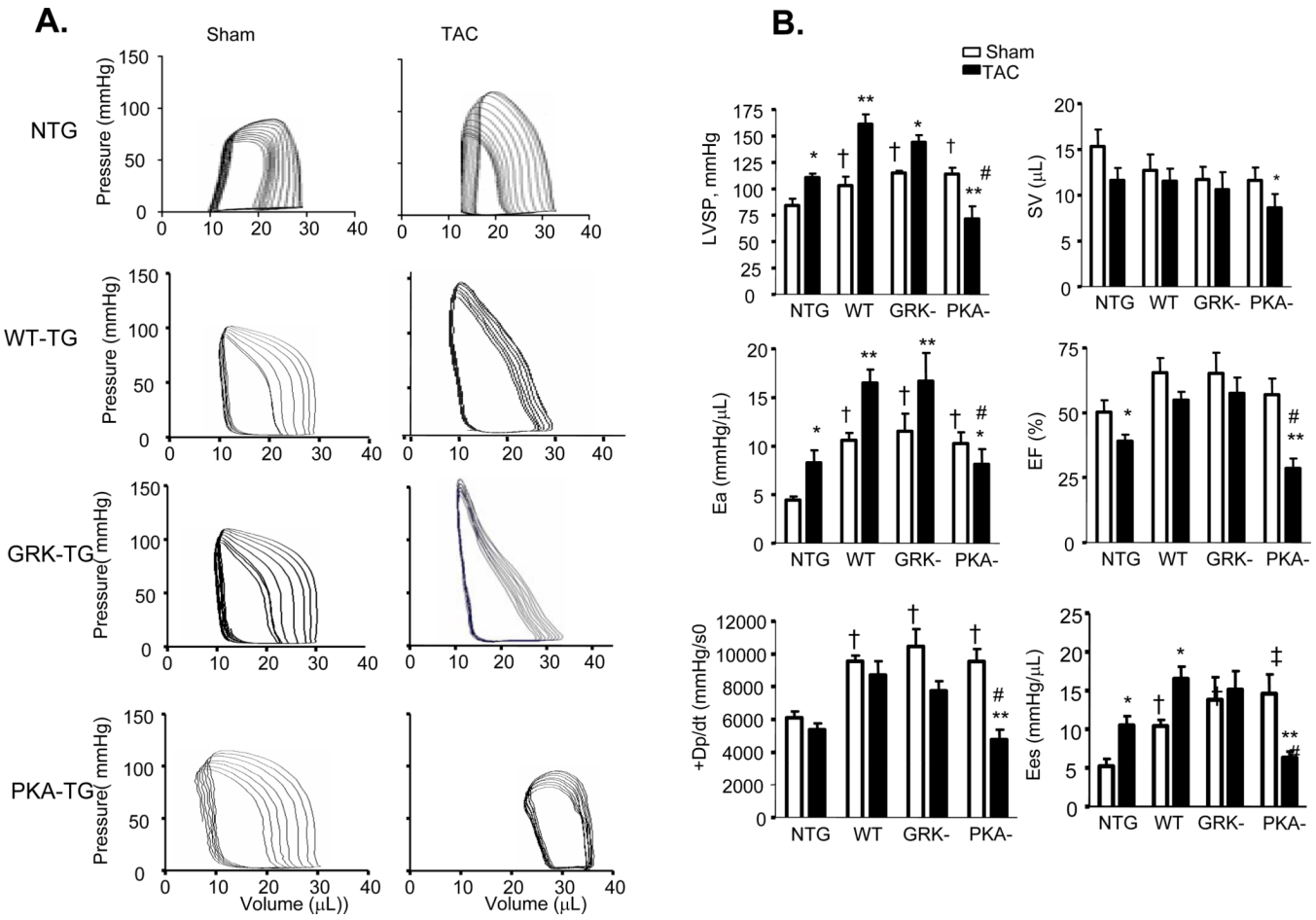


Figure 4. Overexpression of β_2AR mutant lacking PKA phosophorylation sites exaggerated cardiac maladaptive remodeling after pressure overload

(A) Cross sections of hearts subjected to Sham operation (Sham) or TAC for 5 weeks (5w TAC); **(B)** The ratio of heart/body weight (n= 8–12, *p<0.01 *v.s.* Sham; †p<0.01 *v.s.* other three groups with TAC). **(C)** Representative examples of myocardial connective tissue staining (E.P.S. R.) to show cardiac fibrosis (top) and average data of fibrosis area (bottom) (n=5 for each group, *p<0.01 *v.s.* Sham; †p<0.01 *v.s.* other three groups with TAC).



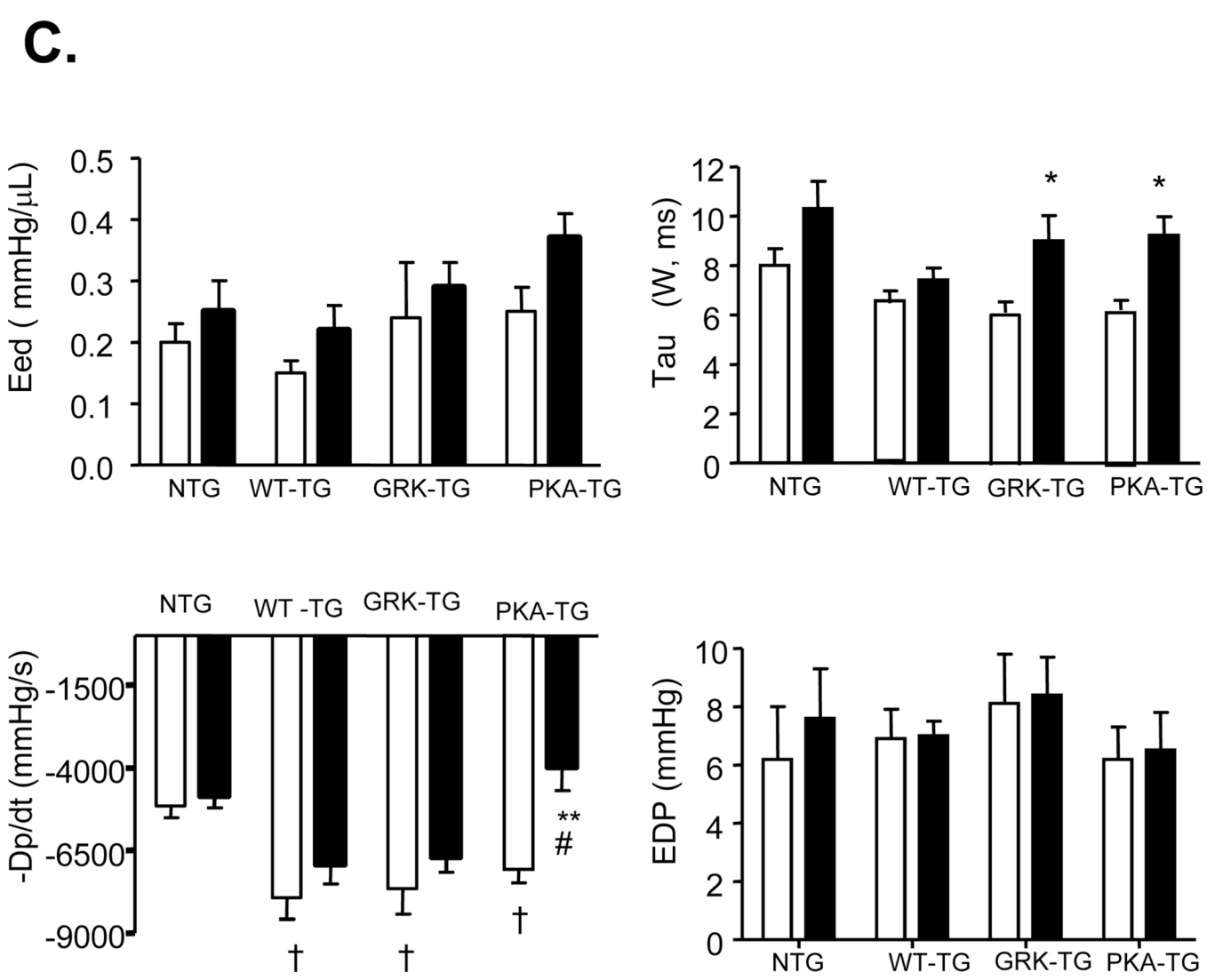
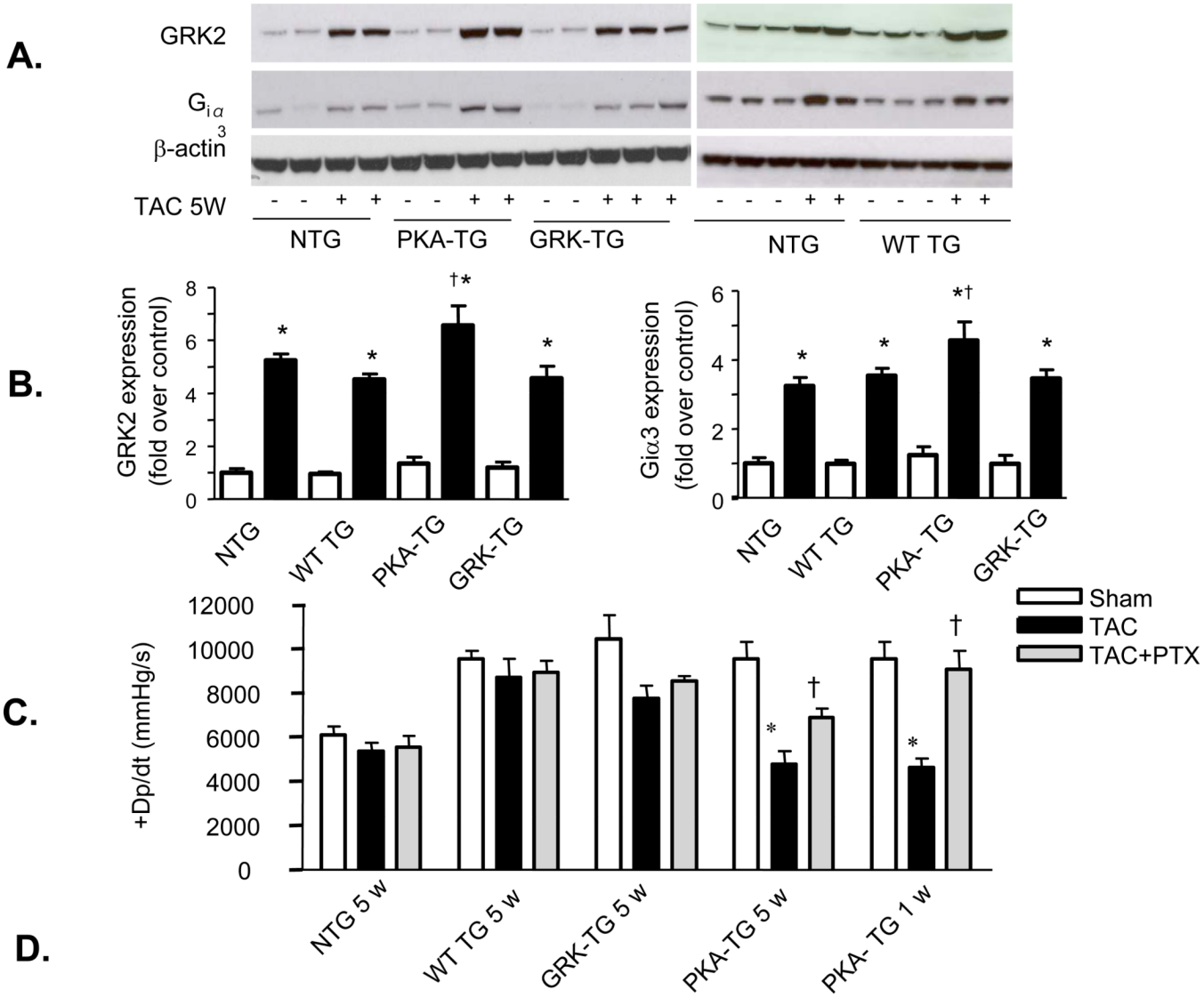


Figure 5. ***In vivo*** **cardiac pressure-volume relations in mice with sham operation or 5 weeks after TAC**

(A) Left-ventricular pressure-volume loops show TAC-induced increase in systolic load. In NTG-, WT TG, GRK- TG mice, TAC leads to rightward shift of the loops and end-systolic pressure-volume relation, marking hypertrophy remodeling. But in PKA- TG mice, TAC induces hear failure (HF). **(B, C)** Summary data on systolic function (B) and diastolic function (C). LVSP, left ventricle systolic pressure. SV, stroke volume. Ea, arterial elastance (measure of ventricular afterload). EF, ejection fraction. +dp/dt, maximum dP/dt. Ees, endsystolic pressure-volume relationship (ESPVR), Eed, end-diastolic pressure-volume relationship (ESDPVR). EDP, end-diastolic pressure. Tau, Regression of Log (pressure) *vs* time (Weiss method). All values present means ± SE (n=8–13 for each group, *P<0.05, **P<0.01 *v.s.* respective Sham; †P<0.05, ‡P<0.01 *v.s.* respective TAC).



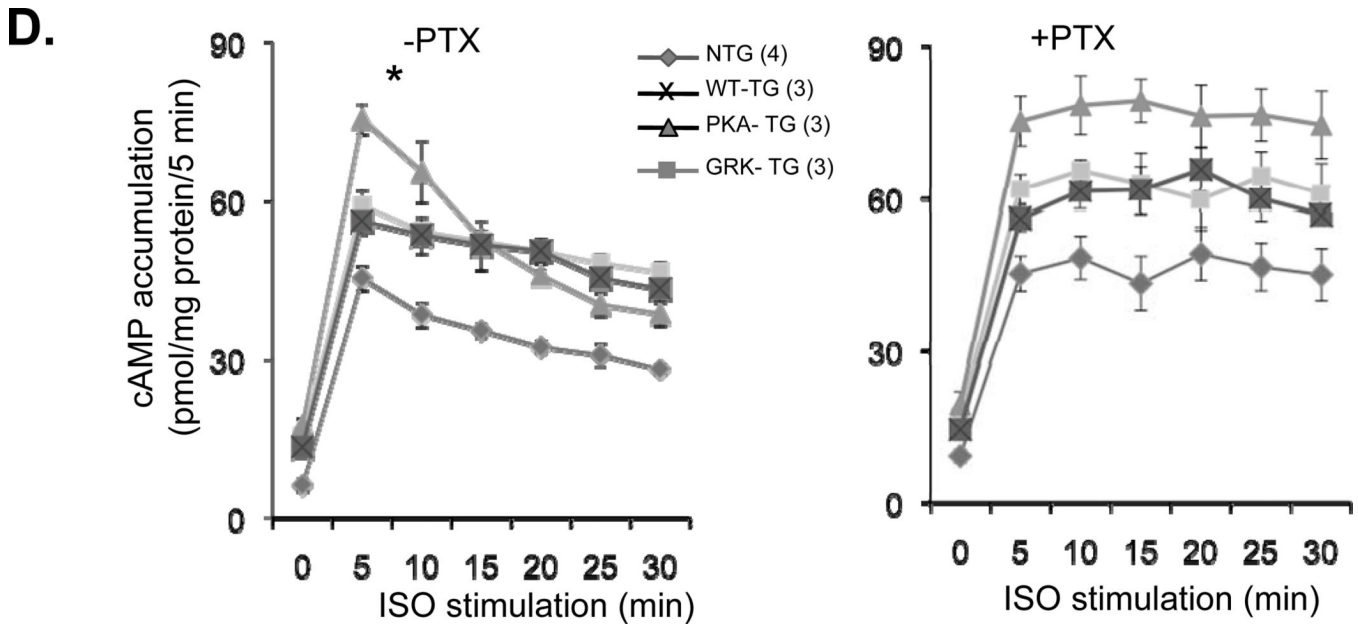


Figure 6. TAC-induced heart failure is associated with greater increases in G_i and GRK2 expression and inhibition of G_i signaling with PTX restores cardiac contractility and blocks the decay of cAMP accumulation by ISO in PKA- TG mouse hearts

(A, B) Panel A shows representative Western bolts using a specific antibody reacting with GRK2 or $G_{i\alpha 3}$ to assay GRK2 and $G_{i\alpha 3}$ protein levels in hearts from NTG, WT TG, PKA-TG or GRK2- TG mice. β-actin was used as a control for protein loading. Panel B displays the average data (n=4–6 for each group, *p<0.01 *v.s.* respective Sham; †p<0.05 *v.s.* other three groups with TAC. **(C)** PTX restored the blunted cardiac contractility in PKA- TG mice subjected to 1 week or 5 weeks TAC (n=5–13, *p<0.05 *v.s.* respective Sham; †P<0.05 *v.s.* respective TAC). Note that PTX treatment fully restored contractile response in the early stage heart failure in PKA- TG mice subjected to 1 week TAC and significantly improved cardiac contractility even in the later stage heart failure in PKA- TG mice after 5 weeks TAC. (**D**) The decay of cAMP accumulation evoked by ISO (left panel) is prevented by PTX treatment (right panel). Cardiomyocytes from NTG, WT-, PKA-, GRK- TG mice were stimulated with isoproterenol (ISO, 10 μM). At each indicated time point, cells were treated with 200 μM of IBMX (3-isobutyl-1-methylxanthine) for 5 minutes before stopping reaction to accumulate cAMP. Note that the decay of cAMP response is accelerated in cardiomyocytes from PKA- TG mice compared to that of other groups, and that this genotype-specific difference is prevented by PTX treatment. *P<0.05 *v.s.* other three strains.