Heart Failure

Cardiac G-Protein–Coupled Receptor Kinase 2 Ablation Induces a Novel Ca\textsuperscript{2+} Handling Phenotype Resistant to Adverse Alterations and Remodeling After Myocardial Infarction

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Background—G-protein–coupled receptor kinase 2 (GRK2) is a primary regulator of β-adrenergic signaling in the heart. G-protein–coupled receptor kinase 2 ablation impedes heart failure development, but elucidation of the cellular mechanisms has not been achieved, and such elucidation is the aim of this study.

Methods and Results—Myocyte contractility, Ca\textsuperscript{2+} handling and excitation-contraction coupling were studied in isolated cardiomyocytes from wild-type and GRK2 knockout (GRK2KO) mice without (sham) or with myocardial infarction (MI). In cardiac myocytes isolated from uninfarcted wild-type and GRK2KO hearts, myocyte contractions and Ca\textsuperscript{2+} transients were similar, but GRK2KO myocytes had lower sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} content because of increased sodium-Ca\textsuperscript{2+} exchanger activity and inhibited SR Ca\textsuperscript{2+} ATPase by local protein kinase A–mediated activation of phosphodiesterase 4 resulting in hypophosphorylated phospholamban. This Ca\textsuperscript{2+} handling phenotype is explained by a higher fractional SR Ca\textsuperscript{2+} release induced by increased L-type Ca\textsuperscript{2+} channel currents. After β-adrenergic stimulation, GRK2KO myocytes revealed significant increases in contractility and Ca\textsuperscript{2+} transients, which were not mediated through cardiac L-type Ca\textsuperscript{2+} channels but through an increased SR Ca\textsuperscript{2+}. Interestingly, post-MI GRK2KO mice showed better cardiac function than post-MI control mice, which is explained by an improved Ca\textsuperscript{2+} handling phenotype. The SR Ca\textsuperscript{2+} content was better maintained in post-MI GRK2KO myocytes than in post-MI control myocytes because of better-maintained L-type Ca\textsuperscript{2+} channel current density and no increase in sodium-Ca\textsuperscript{2+} exchanger in GRK2KO myocytes. An L-type Ca\textsuperscript{2+} channel blocker, verapamil, reversed some beneficial effects of GRK2KO.

Conclusions—These data argue for novel differential regulation of L-type Ca\textsuperscript{2+} channel currents and SR load by GRK2. G-protein–coupled receptor kinase 2 ablation represents a novel beneficial Ca\textsuperscript{2+} handling phenotype resisting adverse remodeling after MI. (Circulation. 2012;125:2108-2118.)

Key Words: calcium ■ experimental models ■ heart failure ■ excitation contraction coupling ■ G-Protein coupled receptor kinase 2

Iterations in myocyte contractility and Ca\textsuperscript{2+} cycling are hallmarks of the progression of heart failure (HF). In failing cardiac myocytes, the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} content is decreased and the Ca\textsuperscript{2+} transient is decreased and prolonged, resulting in depressed myocyte contractility. This is generally considered to be a consequence of increased sodium-Ca\textsuperscript{2+} exchanger (NCX) activity, reduced SR Ca\textsuperscript{2+} ATPase (SERCA) expression and activity (probably due to decreased phospholamban [PLB] phosphorylation), and increased PLB/ SERCA ATPase ratio, as well as an augmented

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open probability of the ryanodine receptor, causing SR Ca\(^{2+}\) leak.\(^2,3\) Alterations of the L-type Ca\(^{2+}\) channel (LTCC), the trigger for the Ca\(^{2+}\)-induced SR Ca\(^{2+}\) release, also contribute to the pathophysiological changes in Ca\(^{2+}\) homeostasis in failing myocytes.\(^4\) In addition to these changes causing dysfunctional contractile performance, the rise of diastolic Ca\(^{2+}\) may increase the risk of arrhythmias and induce pathological cardiac remodeling.\(^5\)

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The increased activity of the sympathetic nervous system associated with HF is a compensation to normalize cardiac function by enhancing Ca\(^{2+}\) cycling and maximize contractile force through the \(\beta\)-adrenergic signaling pathway. In acute HF, these changes can improve systemic perfusion whereas in chronic HF the augmentation in catecholamines is associated with mortality\(^6\) and results in a downregulation of \(\beta\)-adrenergic receptors (\(\beta\)ARs) promoted by upregulated G protein–coupled receptor kinase 2 (GRK2). G protein–coupled receptor kinase 2 is the primary GRK in the heart and a prototype regulator of \(\beta\)AR signaling.\(^7\) We have previously identified GRK2 as a culprit in the progression of HF, and GRK2 inhibition (by expression of its \(\alpha\)-terminal domain, called BARKct) or gene silencing has rescued disparate models of HF.\(^8-10\) Our recent study indicates that the benefits of BARKct could be related to enhanced myocyte contractility by increasing LTCC currents and its responsiveness to \(\beta\)-adrenergic agonists.\(^11\) However, the exact underlying cellular mechanisms for these beneficial effects in HF after BARKct expression or GRK2 ablation are not clearly defined. It is especially important to define these mechanisms because in light of the recent success of \(\beta\)AR blocker therapy in clinical HF management, the results with BARKct and GRK2 silencing appear paradoxical, as the major function of GRK2 in cardiac myocytes is to dampen \(\beta\)AR signaling in a manner similar to that of \(\beta\)AR blockade. We have found that BARKct expression can cause a molecular remodeling of the cardiac \(\beta\)AR system with receptor upregulation and improved \(\beta\)AR signaling, and a recent study with chronic mediated BARKct expression in a rat HF model showed that myocardial \(\beta\)AR changes are probably down-stream of neurohormonal lowering including reduction in sympathetic nervous system activity.\(^12\) In this regard, the role of GRK2 inhibition must mechanistically go beyond resensitizing \(\beta\)ARs and fully understanding GRK2-dependent signaling pathways might enlighten novel therapeutic targets.

To date, little is known about how GRK2 specifically alters cardiac myocyte function and Ca\(^{2+}\) cycling in normal and failing cardiac myocytes. The present study was designed to define the role of GRK2 and GRK2-dependent signaling in excitation-contraction coupling (ECC) in normal and diseased hearts. We used our previously characterized cardiogenic specific GRK2KO mice\(^10,13\) to study myocyte ECC coupling and Ca\(^{2+}\) homeostasis in cardiac myocytes from mice with or without post-MI ischemic HF. We demonstrate for the first time that loss of GRK2 induces a distinct Ca\(^{2+}\) handling phenotype: Myocyte contractility and Ca\(^{2+}\) handling are normal even though the SR Ca\(^{2+}\) content is reduced because there is an increase in LTCC activities and resulting increases in LTCC currents (\(r_{Ca,L}\)) with a compensatory increase in NCX activity. This Ca\(^{2+}\) handling phenotype brought about by GRK2 ablation is resistant to adverse Ca\(^{2+}\) handling remodeling after MI and leads to better cardiac function in GRK2KO mice post MI.

Methods

Conditional mice bearing floxed GRK2 alleles were described previously.\(^10,13\) G-protein–coupled receptor kinase 2 KO (\(\alpha\) myosin heavy chain – Cre-recombinase \(\times\) GRK2flox/flox) and wild-type (WT) (GRK2flox/flox) mice were maintained on a C57BL/6 genetic background. All animal procedures and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Thomas Jefferson University. G-protein–coupled receptor kinase 2 KO and WT mice were 8 to 10 weeks of age when entering the study. Unstressed normal mice and mice with coronary artery ligation (myocardial infarction [MI]) or sham operation were studied. Myocardial infarction was induced by ligating the left anterior descending coronary artery at 2 to 3 mm below its origin as described previously.\(^10,14\) and animals were studied 28 days post-MI or sham operation. For the verapamil study, mice were treated with verapamil starting 14 days after MI or sham operation until the end of the study period (42 days after MI). Verapamil-treated mice received oral supplementation of verapamil (Sigma-Aldrich, St. Louis, MO) as described previously.\(^15\) and were evaluated by echocardiography. Cardiac myocytes were isolated cultured from animals. Myocyte Ca\(^{2+}\) transients and contractions, SR Ca\(^{2+}\) load, \(r_{Ca,L}\), and NCX-Activity \(k_{NCX}\) were measured. Quantitative real-time polymerase chain reaction and Western blot analysis were performed for gene-expression assessment. Detailed description of experimental procedures is available in the online-only Data Supplement.

Data are expressed as mean \(\pm\) SEM. An unpaired 2-tailed t test or a 1-way ANOVA and a 2-way ANOVA (linear mixed effects model) were performed with SAS 9.3 for between-group comparisons, followed by a posthoc Bonferroni adjustment. For all tests, a \(P\) value <0.05 was considered significant.

Results

Myocyte GRK2 Ablation Enhances Adrenergic Responsiveness of Cellular Contractility and Ca\(^{2+}\) Transients

Our previous study has shown that GRK2 KO mice have cardiac function comparable to control mice at baseline (online-only Data Supplement Table 1), but their cardiac function responds better to \(\beta\)-adrenergic stimulation.\(^10\) Here, we determine the cellular mechanisms for this observation. Myocyte contraction and intracellular Ca\(^{2+}\) transients were recorded from WT and GRK2KO myocytes under baseline conditions and after \(\beta\)AR stimulation. Myocytes from both lines revealed a similar fractional shortening when paced at both 0.5Hz and 2Hz (Figure 1A–1C). However, after isoproterenol (ISO), myocytes from GRK2KO mice showed a significantly greater increase in fractional shortening (Figure 1A–1C and online-only Data Supplement Table II). In a good agreement, as shown in Figure 1D, at baseline, the characteristics and amplitude of the Ca\(^{2+}\) transients (as \(\Delta Fura-2\) 340/380 nm ratio) were similar in both groups of myocytes. Stimulation with ISO mediated an anticipated increase in the amplitude of the Fura-2 ratio in WT cardiac myocytes and a greater increase in GRK2KO cardiac myocytes (Figure 1D–1F, online-only Data Supplement Table II). We observed no differences in the Ca\(^{2+}\) transient decay time constants at baseline and after ISO between groups.
GRK2 Silencing in Myocytes Enhances ECC Efficiency

Myocyte contractility and Ca\(^{2+}\) transients are determined by Ca\(^{2+}\) release from the SR.\(^{16}\) We measured SR Ca\(^{2+}\) content by rapid application of caffeine (caffeine spritz) at baseline or after ISO stimulation. Figure 2A shows representative tracings of cytosolic Ca\(^{2+}\) transients (measured with indo-1 AM) induced by caffeine spritz after 4 field stimulations in WT and GRK2KO cardiac myocytes to measure the SR Ca\(^{2+}\) load. At baseline, SR Ca\(^{2+}\) load was less in GRK2 myocytes than in control myocytes (Figure 2B); in response to ISO, the SR Ca\(^{2+}\) load in cardiac myocytes from both WT and GRK2KO mice was significantly increased, but GRK2KO myocytes had a greater increase (Figure 2B). Interestingly, a significantly higher fractional release of Ca\(^{2+}\) was observed in GRK2KO cardiac myocytes compared with WT myocytes at both baseline and after ISO (Figure 2C and online-only Data Supplement Table II).

**Figure 1.** The loss of GRK2 in myocytes enhances the responsiveness of myocyte contraction and Ca\(^{2+}\) transients to βAR stimulation. A, Representative tracings of single myocyte contractions under basal conditions and stimulation with isoproterenol at 0.5 Hz. Averaged myocyte fractional shortening at 0.5 Hz (B) and 2.0 Hz (C) stimulation frequencies under basal conditions and stimulation with isoproterenol. D, Representative intracellular Ca\(^{2+}\) transients measured with Fura-2 (340/380 nm ratio) under basal conditions and stimulation with isoproterenol at 0.5 Hz from the same cell as in A. Averaged Fura-2 ratio amplitude at 0.5 Hz (E) and 2.0 Hz (F) stimulation frequencies under basal conditions and isoproterenol. For measurements represented by B, C, E and F, a total of 48 to 63 cardiac myocytes from 3 different hearts were measured for each group; 2-way ANOVA was used for B, C, E, and F. WT indicates wild type; GRK2KO, G-protein–coupled receptor kinase 2 knockout; and Iso, isoproterenol.
Loss of Myocyte GRK2 Enhances I_{Ca,L} by a Local Protein Kinase A–Dependent Mechanism but Blunts Its βAR Responsiveness

To explore the underlying cellular mechanisms for the reduced SR Ca^{2+} content with enhanced EC coupling efficiency in GRK2 KO myocytes, we measured the I_{Ca,L} in GRK2KO and control myocytes because I_{Ca,L} serves as both the trigger of Ca^{2+} release from the SR and the source for loading the SR.16 Peak I_{Ca,L} density at baseline was significantly greater in GRK2KO compared with WT cardiac myocytes (Figure 3A and 3B and online-only Data Supplement Table II). When stimulated with a saturating dose of ISO (10^{-6} M), peak I_{Ca,L} was increased to the same level in cardiac myocytes from both mouse lines (Figure 3A and 3B), suggesting that the LTCC density was similar in both groups.4 The voltage dependence of channel activation was shifted to more negative voltages in GRK2KO myocytes at baseline, and ISO stimulation caused a significant leftward shift of activation in WT myocytes but not in GRK2KO myocytes (Figure 3C and 3D). These results imply that the LTCC in GRK2KO myocytes could be in such a high activity state that it loses responsiveness to β-adrenergic stimulation.

To explore the underlying mechanisms for these changes of I_{Ca,L} properties, we tested whether the increased I_{Ca,L} was due to increased available channels on the surface membrane, the increase of channel activities at single-channel levels, or both. Charge movement was used to quantify the number of available channels on the surface membrane of KO and control WT myocytes. Figure 3E shows that there was no significant difference in the charge movements of the LTCC induced by various depolarizing voltages, a result which indicates that there was no significant alteration of LTCC density on the membrane and that the increased whole-cell I_{Ca,L} could be due to the increased single-channel activities. Immunoprecipitation of α1c, the pore-forming subunit of the LTCC, from the same amount of proteins in GRK2KO and control hearts showed no difference in α1c expression (Figure 3N). These data suggest that the LTCC in GRK2KO myocytes must have higher than normal activity, a result supported by single-channel recording of LTCC activities (Figure 3J–3M). The availability (Figure 3K) and the open probabilities (Figure 3L and 3M) were significantly increased in GRK2KO myocytes, a result that could fully explain the increase in whole-cell I_{Ca,L} in KO myocytes.

Enhanced phosphorylation of the LTCC by protein kinase A (PKA) may result in increased LTCC activity.4,17 Previously, we have shown that βARs carry constitutive activation to activate PKA locally to phosphorylate the LTCC.16 Here, we tested whether high LTCC activity was mediated by a PKA-dependent mechanism. H89, a PKA-specific inhibitor, was used, and it normalized the current density and voltage-dependent activation of the LTCC in KO myocytes but had no significant effect on LTCCs in WT myocytes (Figure 3F–3I). The single-channel study further confirmed that the heightened LTCC activity in the KO myocytes was due to PKA activation because H89 also normalized the increased channel activity in KO myocytes (Figure 3J–3M). The phosphorylation of α1c at Ser1928, a PKA site, was shown to be greater in GRK2KO hearts than in control hearts (Figure 3N and 3O).

Figure 2. GRK2 silencing in cardiac myocytes alters SR Ca^{2+} content and its regulation. A, Representative tracings of the caffeine-induced intracellular Ca^{2+} transients determined with Indo-1 in a control myocyte and a GRK2KO myocyte. B, Caffeine-induced peak intracellular Ca^{2+} amplitudes under baseline conditions and isoproterenol stimulation. C, Fractional release calculated as the ratio of peak Ca^{2+} concentration induced by field stimulation to peak Ca^{2+} concentration induced by caffeine. For measurements represented by figures B through C, a total of 16 to 29 cardiac myocytes from 3 different hearts were analyzed per group; 2-way ANOVA with posthoc Student t test for B and C. WT indicates wild type; GRK2KO, G-protein–coupled receptor kinase 2 knockout; NLC, non littermate control; KO, knockout; and ISO, isoproterenol.

Loss of GRK2 in Myocytes Increases Na^{+}/Ca^{2+}-Exchanger Expression

Because there is a decreased SR Ca^{2+} content with increased I_{Ca,L} in GRK2KO myocytes, there should be increased Ca^{2+} efflux out of GRK2KO myocytes. In ventricular myocytes, the major route of Ca^{2+} efflux is through the NCX. One way to measure NCX activity is to examine the decay rate of caffeine-induced Ca^{2+} transients that can be fit by a single exponential decay equation.19 The tau value was significantly smaller in the GRK2KO myocytes than in control myocytes, and ISO did not change these values (Figure 4A), suggesting that the NCX...
activity at baseline is increased in GRK2KO myocytes compared with WT myocytes, a result that was confirmed by direct measurement of NCX current (Figure 4B) and NCX protein expression (Figure 4C and 4D).

**Loss of GRK2 From Myocytes Decreases Basal PLB Phosphorylation but Increases Its Responsiveness to βAR Stimulation**

The loading of the SR with Ca\(^{2+}\) depends on the competition between the extrusion of Ca\(^{2+}\) out of the cell (mainly through NCX) and the resequestration of Ca\(^{2+}\) into SR by SERCA, which is regulated by PLB.\(^{16}\) Dephosphorylated PLB exerts a tonic inhibition on SERCA activity. For these reasons, we determined the expression level of SERCA and PLB as well as the phosphorylation level of PLB. The expression of SERCA and PLB was not significantly altered by silencing GRK2 (Figure 4E–4H). However, the phosphorylation of PLB at Ser16, a PKA site, was significantly reduced in GRK2KO hearts but the phosphorylation of PLB at Thr17 site was not altered (Figure 4G and 4H). When myocytes were stimulated with the βAR agonist ISO, a robust increase in the phosphorylation of PLB at Ser16 sites was observed in both WT and GRK2KO cardiac myocytes (Figure 4I and 4J). However, a significant leftward shift of the dose–response curve was found in GRK2KO myocytes compared with WT cells (Figure 4J), demonstrating higher βAR sensitivity in cells isolated from unstressed GRK2KO mice.

To further clarify the mechanism responsible for the hypophosphorylation of PLB, we examined whether phosphodiesterase 4 (PDE4), which is activated by PKA, could...
A PDE4-specific inhibitor, rolipram (10 mg/kg BW, i.p.), was injected into unstressed WT and GRK2KO mice for 2 hours to allow it to take effect. Then the hearts were snap frozen in liquid nitrogen, and Western blotting for phospho-PLB and total PLB was performed. Interestingly, rolipram blunted the hypophosphorylation of PLB in GRK2KO myocytes (Figure 4K and 4L), which indicates that the proposed higher PKA activity in GRK2KO myocytes results in an increased local PDE4 activity causing hypophosphorylation of PLB.

Loss of Myocyte GRK2 Before MI Prevents the Development of Heart Failure and Preserves Contractility of Myocytes

Our previous studies have shown that cardiac specific loss of GRK2 ameliorates the development of HF after MI.10 In vivo cardiac function as assessed by echocardiography 28 days after MI showed that although sham GRK2KO mice were indistinguishable from sham WT mice, GRK2KO mice had significantly improved post-MI cardiac function and ventricular remodeling after the loss of myocyte GRK2 (online-only Data Supplement Table I). Because this improved post-MI cardiac function was seen in GRK2KO mice with similar infarct sizes, the beneficial effects of GRK2 deficiency probably occurs at the myocyte level. Therefore, the function of cardiac myocytes isolated from WT and GRK2KO mice at 28 days after MI was determined. The basal fractional shortening and Ca2+/H11001 transient amplitudes in myocytes from GRK2KO mice after MI (GRK2KO MI) were almost normal compared with GRK2KO myocytes from mice not subject to MI and greater than those of WT cardiac myocytes after MI (WT MI) at both pacing frequencies of 0.5Hz and 2Hz (Figure 5A, B, D, and E and online-only Data Supplement Table II). Furthermore, WT MI myocytes had a blunted functional response to ISO. In contrast, cardiac myocytes from GRK2KO mice after MI displayed significantly improved β-adrenergic responses (Figure 5A, B, D, and E). These results clearly show that the loss of GRK2 in cardiac myocytes can partially prevent pathological cellular mechanical and Ca2+/H11001-handling remodeling after MI and provide a potential cellular mechanism for the benefits of GRK2 lowering or inhibition in the failing heart.

Figure 4. Silencing of GRK2 in cardiac myocytes increased NCX expression, reduced PLB phosphorylation by PKA-dependent PDE4 activation, and enhanced responsiveness to ISO. A, Tau values of caffeine-induced intracellular Ca2+ transient decay, indicating increased NCX activity in GRK2KO myocytes; n=16 to 29 cardiac myocytes from 3 different hearts. B, NCX currents at baseline in control (WT) and GRK2KO myocytes; n=4 animals/group, 2-way repeated ANOVA with posthoc Student t test. C, Western blot of NCX proteins in WT and GRK2KO hearts. D, NCX expression normalized to GAPDH. E and F, SERCA expression in WT and GRK2KO hearts. G and H, PLB expression and phosphorylation at Ser16 (PKA site) and Thr17 (CaMK II site) in WT and GRK2KO hearts; n=5 animals/group; data were analyzed by regression with repeated measures for J. K and L, Western blotting for pSer16-PLB and total PLB in WT and GRK2KO hearts stimulated with or without the selective PDE4 inhibitor rolipram; a total of 3 to 5 hearts were analyzed for each group; 1-way ANOVA for L. WT indicates wild type; KO, knockout; ISO, isoproterenol; NCX, Na+/Ca2+ exchange current; NCX1, Na+/Ca2+ exchange 1; SERCA, sarcoplasmic/endoplasmic reticulum Ca2+ ATPase; PLB, phospholamban; PLBt, total Phospholamban; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and N.S., not significant.
GRK2KO MI Myocytes Have Preserved SR Ca$^{2+}$ Loading and $I_{\text{Ca,L}}$

A decrease in the SR Ca$^{2+}$ load is a contributing factor for depressed myocyte contractility, a hallmark of HF. Although GRK2KO mice without MI having a lower SR Ca$^{2+}$ load than WT mice without MI, the SR Ca$^{2+}$ load in GRK2KO MI myocytes was not decreased as in WT MI myocytes (Figure 6A and B and online-only Data Supplement Table II). Fractional Ca$^{2+}$ release from the SR was higher in GRK2KO MI myocytes as well, explaining the preserved myocyte contractility (Figure 6C). Na$^+$/Ca$^{2+}$ exchanger activity, as indirectly assessed by the decay constant tau of the caffeine-induced Ca$^{2+}$ transient was normalized in post-MI GRK2KO myocytes, despite significant increases in NCX activity in infarcted WT mice consistent with severe HF (Figure 6D).

Improvements in intracellular Ca$^{2+}$ transients and SR fractional Ca$^{2+}$ release in the GRK2KO MI myocytes could result from changes in cardiac myocyte $I_{\text{Ca,L}}$. Although basal $I_{\text{Ca,L}}$ amplitudes in both post-MI WT and GRK2KO myocytes were reduced compared with pre-MI values (see Figure 3A and 3B), peak $I_{\text{Ca,L}}$ in post-MI myocytes were significantly greater with the loss of GRK2 (Figure 6E and online-only Data Supplement Table II). When stimulated with ISO (10$^{-6}$ M), $I_{\text{Ca,L}}$ in GRK2KO post-MI myocytes was only insignificantly increased (12.9±6.9%, n=4), but $I_{\text{Ca,L}}$ in post-MI WT myocytes was significantly increased by 84.3±18.1% (n=7) (Figure 6F). However, after ISO, $I_{\text{Ca,L}}$ amplitudes in myocytes were not different between the 2 mouse lines (Figure 6G and 6H), indicating similar LTCC density. The marked enhancement in basal $I_{\text{Ca,L}}$ in the GRK2KO MI myocytes might contribute to the normalization of intracellular Ca$^{2+}$ handling and improved cardiac myocyte contractility. Of interest, the ISO stimulation caused a significant leftward shift of voltage dependency of channel activation in WT post-MI myocytes but not in GRK2KO myocytes, probably because the activation of $I_{\text{Ca,L}}$ in GRK2KO MI myocytes at baseline was already shifted to the left. After ISO stimulation, the voltage-dependent activation of $I_{\text{Ca,L}}$ was similar in both groups (Figure 6G and 6H).

Loss of Myocyte GRK2 Inhibits Adverse Cellular Remodeling Post-MI

Cardiac myocyte size at 28 days post-MI was assessed by measurements of myocyte capacitance. Myocytes isolated from GRK2KO MI mice had significantly smaller capacitance, indicating less myocyte hypertrophy and inhibition of adverse cellular remodeling compared with WT mice post-MI (GRK2KO MI 211±19pF versus WT MI 311±35pF versus WT Sham 178±19pF versus KO Sham 188±15pF; $P<0.05$ between GRK2KO MI and WT MI).

Figure 5. Loss of GRK2 in cardiac myocytes ameliorates single-cell contractility and Ca$^{2+}$ handling post-MI. Measurements were obtained at 0.5Hz (A–C) or 2Hz (D–F) under basal conditions and isoproterenol stimulation (10$^{-8}$ mol/L). Fractional shortenings (A and D), Fura-2 ratio amplitudes (B and E) representing the change of intracellular Ca$^{2+}$ transient from baseline, and tau of Ca$^{2+}$ transient decay (C and F) were shown. For all measurements, a total of 60 cardiac myocytes from 6 different hearts were measured for each group; 2-way ANOVA. Iso indicates isoproterenol; MI, myocardial infarction; WT, wild type; and GRK2KO, G-protein–coupled receptor kinase 2 knockout.
Beneficial Effects of Myocyte GRK2 Silencing Is Suppressed by the LTCC Blocker Verapamil

To determine if the beneficial Ca\textsuperscript{2+}/H\textsubscript{11001} effects seen post-MI after myocyte GRK2 lowering is mediated through the novel changes in LTCC function, we treated WT and GRK2KO mice with verapamil from 14 days till 42 days post-MI. Interestingly, echocardiography revealed that verapamil treatment negated some of the beneficial effects of GRK2 silencing on post-MI cardiac function whereas the LTCC blocker had minimal effects on post-MI WT mice (Figure 7A). Furthermore, cardiac brain natriuretic peptide messenger RNA expression as a molecular marker of HF was significantly lower in post-MI GRK2KO mice compared with WT MI mice but was reversed by verapamil treatment to the level seen in post-MI WT mice (Figure 7C). It could be true that the absolute amount of Ca\textsuperscript{2+} current blocked by verapamil is more in GRK2 KO myocytes because the total LTCC density is higher in GRK2 KO MI myocytes, and thus verapamil had a stronger effect in KO MI myocytes. In conclusion, it appears that the beneficial effects offered by a loss of GRK2 in cardiomyocytes are at least in part attributable to the upregulation of ICa,L.

Discussion

G-protein–coupled receptor kinase 2 is an important molecule in the heart. It is not only a primary regulator of adrenergic signaling but also claims an important role in the development of HF.8–10 It is upregulated during the early stage in injured myocardium, indicating that it participates in the progression of ventricular dysfunction and cardiomyopathy.8–10 Our previous studies have shown that GRK2 silencing or inhibition by βARKct is able to improve cardiac function during HF progression after MI. However, the specific role of GRK2 in the regulation of normal and failing Ca\textsuperscript{2+}/H\textsubscript{11001} cycling has never been studied. In HF, myocyte Ca\textsuperscript{2+}/H\textsubscript{11001} cycling is deranged, and abnormalities include altered cardiac LTCC density and properties and reduced SR Ca\textsuperscript{2+}/H\textsubscript{11001} content due to decreased SERCA and increased NCX activities. These changes result in reduced intracellular Ca\textsuperscript{2+}/H\textsubscript{11001} transients and depressed myocyte contractility.1,4 Our current study has revealed that GRK2 can influence myocyte Ca\textsuperscript{2+} handling phenotype that is resistant to cardiac function deterioration after MI. The benefits rendered by GRK2 silencing are associated with the differential regulation of sarcolemmal and SR Ca\textsuperscript{2+}/H\textsubscript{11001} handling by the β-adrenergic system.

A Novel Ca\textsuperscript{2+}/H\textsubscript{11001}-Handling Phenotype Induced by GRK2 Silencing

Although GRK2 plays an important role in regulating the β-adrenergic system, its loss does not affect basal cardiac and cardiomyocyte function. Myocyte loss of GRK2 did not change characteristics of basal intracellular Ca\textsuperscript{2+}/H\textsubscript{11001} transients and myocyte contractions. However, detailed characterization of myocyte Ca\textsuperscript{2+}/H\textsubscript{11001} handling has shown many differences in EC coupling between GRK2KO and WT myocytes: (1) The SR Ca\textsuperscript{2+}/H\textsubscript{11001} content is reduced in GRK2 KO myocytes, but Ca\textsuperscript{2+}/H\textsubscript{11001}...
transients and contraction in GRK2KO myocytes are normal because of an increased fractional Ca\(^{2+}\) release from the SR; (2) increased \(I_{Ca,L}\) ensures normal Ca\(^{2+}\) transients and cardiac myocyte contractility; (3) decreased SR Ca\(^{2+}\) content in the face of increased \(I_{Ca,L}\) is due to increased Ca\(^{2+}\) efflux through the NCX and the inhibition of SERCA by hypophosphorylated PLB; and (4) increased \(I_{Ca,L}\) is possibly due to local increase in PKA activity. Most of these aspects of Ca\(^{2+}\) handling in GRK2KO myocytes, except the greater than normal \(I_{Ca,L}\) and enhanced \(-\)adrenergic regulation, have some similarity with those observed in failing myocytes\(^{21}\). These findings could imply that, even in failing myocytes, some of the Ca\(^{2+}\) handling aspects could be more of adaptive mechanisms.

**Differential Regulation of Sarcolemmal and SR Ca\(^{2+}\) Handling by the \(-\)Adrenergic System**

Our data clearly show that there is a differential regulation of the LTCC on the sarcolemma and the PLB on the SR by the \(-\)adrenergic system in GRK2KO myocytes: At baseline, the LTCC is already in high-activity mode probably because of the high-phosphorylation state of the channel, but the LTCC loses its responses to \(-\)adrenergic stimulation; in contrast, the PLB is in a low-phosphorylation state (hypophosphorylation), but it has enhanced responsiveness to \(-\)adrenergic receptor (\(-\)AR) stimulation. Our study indicates that the increased LTCC activity could be due to an increase in subsarcolemmal (local) PKA activation brought about by constitutive activity of the \(-\)ARs. In normal cardiac physiology, GRK2 mediates the desensitization of \(-\)ARs\(^{20}\). The loss of GRK2 prevents desensitization of \(-\)ARs and thus likely promotes the accumulation of activated \(-\)ARs in GRK2KO myocytes even after the isolation. The increase in LTCC activity induced by constitutive \(-\)ARs has been shown in cardiac \(-\)AR–overexpression mice\(^{18}\). The high LTCC activity in GRK2KO myocytes blunts the responsiveness of the channel to \(-\)adrenergic stimulation. Similar situations have been reported in myocytes with high basal LTCC activities\(^{17,22}\). Recently, we have shown that overexpression of \(\beta\)-ARKct, an inhibitor of GRK2, in adult rat myocytes increases basal \(I_{Ca,L}\), as we have seen with GRK2 silencing. However, \(\beta\)-ARKct overexpression also enhanced the responses of \(I_{Ca,L}\) to ISO\(^{11}\), which is in contrast to our findings with GRK2KO. These results suggest that potentially different mechanisms are involved in our current study and the \(\beta\)-ARKct study\(^{11}\), with the net effect (increased LTCC) being comparable. Primarily, \(\beta\)-ARKct reduces the inhibitory effect of the \(\beta\)-subunits of activated heterotrimeric-G proteins (G\(_{\beta\gamma}\)) on the LTCC whereas GRK2KO leads to a local increase in PKA, thereby activating the LTCC. The role of G\(_{\beta\gamma}\) in this setting was not specifically addressed, however: If G\(_{\beta\gamma}\) was released with the KO of GRK2, the inhibitory effect on the LTCC must be at least overcome by the PKA-dependent activation of the LTCC. An interesting experiment for future studies will indeed be the expression of \(\beta\)-ARKct in GRK2KO myocytes.

**Figure 7.** Beneficial effects of myocytes’ GRK2 knockout are reduced by the LTCC blocker verapamil. Echocardiographic measurements of fractional shortening (A) and left ventricular end-diastolic diameter (B) at 42 days post-MI or sham operation. Mice were supplemented orally with verapamil starting 14 days post-MI until the end of the study period (42 days post-MI). n=6 to 8/sham group; n=15 to 25/MI group without verapamil; n=10 to 13/MI group with verapamil; 1-way ANOVA. C, Cardiac myocyte brain natriuretic protein messenger RNA levels 42 days post-MI or sham operation; n=5 to 6/group for sham; n=8 to 9/group for MI; 1-way ANOVA and unpaired 2-tailed t test. FS indicates fractional shortening; WT, wild type; GRK2KO, G-protein–coupled receptor kinase 2 knockout; MI, myocardial infarction; EDD, end-diastolic diameter; BNP, brain natriuretic protein; and mRNA, messenger RNA.
This experiment will finally address the role of $G_{i\alpha}$ in this setting, but such a goal goes far beyond the scope of our current study. The use of different models (GRK2KO in mice in vivo for a relatively long period of time versus $\beta$ARKct expression in cultured rat ventricular myocytes$^{11}$ in vitro for 24 hours) could also account for different mechanisms in mediating increased basal $I_{Ca,L}$ and different degrees of responsiveness to ISO stimulation.

In contrast to the enhanced LTCC phosphorylation in GRK2KO myocytes, the phosphorylation state of PLB on the SR is lower than normal and the responsiveness of PLB to $\beta$-adrenergic agonists is enhanced. The underlying mechanism is related to locally activated PDE4 by activated subsarcolemmal PKA because rolipram, a selective PDE4 inhibitor, blunted the hypophosphorylation of PLB in GRK2KO hearts. Subsarcolemmal PDEs are generally able to diffuse to the SR and thus can limit local cAMP production and PKA activation.$^{23}$ Our results mechanistically explain the differential regulation of sarcolemmal versus SR Ca$^{2+}$ handling associated with the cardiac myocyte lowering of GRK2, which ultimately can improve cardiac function in HF models.

The Novel Ca$^{2+}$-Handling Phenotype Induced by the Loss of GRK2 Is Resistant to Adverse Remodeling in Hearts After MI

As summarized above, GRK2KO induces a novel Ca$^{2+}$-handling phenotype that maintains a normal myocyte contractility in a way that is less dependent on SR Ca$^{2+}$ but more dependent on $I_{Ca,L}$. We also show that this type of Ca$^{2+}$ handling in GRK2KO myocytes is more resistant to adverse remodeling induced by MI in that the SR Ca$^{2+}$ content and its regulation by the $\beta$AR system and $I_{Ca,L}$ density are better preserved and NCX activity is not increased. The cellular processes responsible for better remodeling after MI in GRK2KO mice are potentially due to a combination of increased $I_{Ca,L}$ and normalized NCX activity resulting in reduced SR Ca$^{2+}$ content at baseline. We suspect that the relatively unchanged and small NCX activity after MI predisposes the GRK2KO MI myocyte to maintain an unchanged SR Ca$^{2+}$ loading, which is in contrast to WT mice. Overall, this combination renders cardiac myocytes less susceptible to SR Ca$^{2+}$ overload, which is known to induce myocyte apoptosis and necrosis.$^{24,25}$ Importantly, the beneficial effects of GRK2KO are negated by an LTCC antagonist, verapamil. Elevated SR Ca$^{2+}$ content might also participate in myocyte hypertrophy.$^{26}$ In this study, GRK2KO myocytes develop less cardiac myocyte hypertrophy post-MI. This could be due to the concomitant decrease of SR Ca$^{2+}$ load induced by the loss of GRK2 expression in the cardiac myocyte. The enhanced $\beta$-adrenergic responsiveness in GRK2KO myocytes may also contribute to the beneficial effects of GRK2 silencing.

The results obtained here explain our previous studies showing that the loss of GRK2 in cardiac myocytes reduces HF-associated mortality and enhances global cardiac function post-MI.$^{10}$ The improvements associated with the loss of GRK2 expression and activity are in large part attributable to the normalization of intracellular Ca$^{2+}$ cycling and cardiac myocyte function.

Conclusions

In summary, our data provide novel and important insights into the role of GRK2 in normal and failing hearts. Loss of GRK2 in cardiac myocytes enhances ECC efficiency in the presence of a lower than normal SR Ca$^{2+}$ loading condition and better $\beta$-adrenergic responsiveness in unstressed hearts. This enhancement of ECC occurs through differential regulation of sarcolemmal versus SR Ca$^{2+}$ handling, with the net result being improved Ca$^{2+}$ transients leading to the amelioration of the HF phenotype. This is seen at the myocyte level and also globally in vivo with improved cardiac function of GRK2KO mice post-MI. Our data revealed for the first time that the beneficial effects seen with a loss of myocyte GRK2 activity after MI were associated with marked amelioration of cardiac myocyte contractility and significant improvements in intracellular Ca$^{2+}$ cycling effected by modulation of $I_{Ca,L}$. These effects seen at the myocyte level may also contribute to the beneficial effects seen in various HF models treated with the $\beta$ARKct peptide as a GRK2 inhibitor.$^{8,9,12}$ Further, GRK2 appears to induce novel regulatory modulation in the LTCC because currents were enhanced with a loss of GRK2. Overall, our current results mechanistically explain the beneficial effects of GRK2 silencing or inhibition after MI in the heart at the cellular and molecular levels and validates GRK2 as a potential target for HF prevention and treatment.

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Disclosures

None.

References

1. Piacentino V III, Weber CR, Chen X, Weisser-Thomas J, Margulies KB, Bers DM, Houser SR. Cellular basis of abnormal calcium transients of failing human ventricular myocytes. Circ Res. 2003;92:651–658.
2. Houser SR, Piacentino V III, Weisser J. Abnormalities of calcium cycling in the hypertrophied and failing heart. J Mol Cell Cardiol. 2000;32:1595–1607.
3. Bers DM. Altered cardiac myocyte Ca regulation in heart failure. Physiolog (Bethesda). 2006;21:380–387.
4. Chen X, Piacentino V III, Furukawa S, Goldman B, Margulies KB, Houser SR. L-type Ca$^{2+}$ channel density and regulation are altered in failing human ventricular myocytes and recover after support with mechanical assist devices. Circ Res. 2002;91:517–524.
5. Bers DM. Calcium cycling and signaling in cardiac myocytes. Annu Rev Physiol. 2008;70:23–49.
6. Cohn JN, Levine TB, Olivari MT, Garber V, Lora D, Francis GS, Simon AB, Rector T. Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. N Engl J Med. 1984;311:819–823.
7. Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function. Nature. 2002;415:206–212.
8. Harding VB, Jones LR, Lefkowitz RJ, Koch WJ, Rockman HA. Cardiac beta ARK1 inhibition prolongs survival and augments beta blocker therapy in a mouse model of severe heart failure. Proc Natl Acad Sci U S A. 2001;98:5809–5814.
9. Rockman HA, Chien KR, Choi DJ, Iacarino G, Hunter JJ, Ross J Jr, Lefkowitz RJ, Koch WJ. Expression of a beta-adrenergic receptor kinase inhibitor prevents the development of myocardial failure in gene-targeted mice. Proc Natl Acad Sci U S A. 1998;95:7000–7005.

10. Raake PW, Vinge LE, Gao E, Boucher M, Rengo G, Chen X, DeGeorge BR Jr, Matkovich S, Houser SR, Most P, Eckhart AD, Dorn GW II, Koch WJ. G-protein-coupled receptor kinase 2 ablation in cardiac myocytes before or after myocardial infarction prevents heart failure. Circ Res. 2008;103:413–422.

11. Volkers M, Weidenhammer C, Herzog N, Qiu G, Spaich K, von Wegner F, Peppel K, Muller OJ, Schinkel S, Rabinowitz JE, Hippe HJ, Brinks H, Katsum HA, Koch WJ, Eckhart AD, Friedrich O, Most P. The inotropic peptide betaARKct improves βAR responsiveness in normal and failing cardiomyocytes through g(βγ)-mediated L-type calcium current disinhibition. Circ Res. 2011;108:27–39.

12. Rengo G, Lymperopoulos A, Zincarelli C, Donniacuo M, Soltys S, Rabito C, Lohse MJ, Eckhart AD, Dorn GW II, Koch WJ. Myocardial adeno-associated virus serotype 6-betaARKct gene therapy improves cardiac function and normalizes the neurohormonal axis in chronic heart failure. Circulation. 2009;119:89–98.

13. Matkovich SJ, Diwan A, Klanje JL, Hammar DJ, Marcez Y, Odly AM, Brunskill EW, Koch WJ, Schwartz R, Dorn GW II. Cardiac-specific ablation of G-protein receptor kinase 2 redefines its roles in heart development and beta-adrenergic signaling. Circ Res. 2006;99:996–1003.

14. Most P, Seifert H, Gao E, Funakoshi H, Volkers M, Heisterhorst J, Remppis A, Pfeifer ST, DeGeorge BR Jr, Eckhart AD, Feldman AM, Koch WJ. Cardiac S100A1 protein levels determine contractile performance and propensity toward heart failure after myocardial infarction. Circulation. 2006;114:1258–1268.

15. Cohn RD, Dubee J, Moore SA, Coral-Vazquez P, Prouty S, Campbell KP. Prevention of cardiomyopathy in mouse models lacking the smooth muscle sarcoglycan-sarcospan complex. J Clin Invest. 2001;107:R1–R7.

16. Bers DM. Cardiac excitation-contraction coupling. Nature. 2002;415:198–205.

17. Schroder F, Hauk F, Beuckelmann DJ, Hirt S, Hulmin R, Priese L, Schwingher RH, Wei J, Herzig S. Increased availability and open probability of single L-type calcium channels from failing compared with nonfailing human ventricle. Circulation. 1998;98:969–976.

18. Foerster K, Kaeferstein T, Groner F, Engelhardt S, Matthes J, Koch WJ, Lohse MJ, Herzig S. Calcium channel function and regulation in beta 1- and beta 2-adrenoceptor transgenic mice. Naunyn Schmiedebergs Arch Pharmacol. 2004;369:490–495.

19. Tang M, Zhang X, Li Y, Guan Y, Ai X, Szeto C, Nakayama H, Zhang H, Ge S, Molkentin JD, Houser SR, Chen X. Enhanced basal contractility but reduced excitation-contraction coupling efficiency and beta-adrenergic reserve of hearts with increased cav1.2 activity. Am J Physiol Heart Circ Physiol. 299:H519–H528.

20. Rengo G, Lymperopoulos A, Lesosco D, Koch WJ. GRK2 as a novel gene therapy target in heart failure. J Mol Cell Cardiology. 2011;50:785–792.

21. Houser SR, Margulies KB. Is depressed myocardial contractility centrally involved in heart failure? Circ Res. 2003;92:350–358.

22. Miriyala J, Nguyten Y, Yue DT, Colecraft HM. Role of CaVbeta subunits, and lack of functional reserve, in protein kinase A modulation of cardiac CaV1.2 channels. Circ Res. 2008;102:e54–e64.

23. De Arcangelis V, Liu S, Zhang D, Soto D, Xiang YK. Equilibrium between adenyl cyclase and phosphodiesterase patterns adrenergic agonist dose-dependent spatiotemporal cAMP/ protein kinase A activities in cardiomyocytes. Mol Pharmacol. 2010;78:340–349.

24. Math JN, Bodil I, Lewis W, Varadi G, Schwartz A, Ca2+-dependent transgenic model of cardiac hypertrophy: a role for protein kinase Calpha. Circulation. 2001;103:140–147.

25. Nakayama H, Chen X, Baines CP, Klevisky R, Zhang X, Zhang H, Jaleel N, Chua BH, Hewett TE, Robbins J, Houser SR, Molkentin JD. Ca2+- and mitochondrial-dependent cardiomyocyte necrosis as a primary mediator of heart failure. J Clin Invest. 2007;117:2431–2444.

26. Chen X, Nakayama H, Zhang X, Ai X, Harris DM, Tang M, Zhang H, Szeto C, Stockbower K, Berretta RM, Eckhart AD, Koch WJ, Molkentin JD, Houser SR. Calcium influx through CaV1.2 is a proximal signal for pathological cardiomyocyte hypertrophy. J Mol Cell Cardiol. 2011;50:460–470.

**CLINICAL PERSPECTIVE**

G-protein–coupled receptor kinase 2 (GRK2) is a molecular culprit in the development and progression of heart failure (HF). We now provide the molecular basis for potential benefits of therapeutic strategies aiming at GRK2 inhibition. With our current study we demonstrate that loss of GRK2 in cardiac myocytes enhances excitation-contraction coupling in unstressed hearts and in failing myocytes. We show that this enhancement of excitation-contraction coupling occurs through differential regulation of sarcoplasmic reticulum Ca2+ handling, with the net result being improved Ca2+ transients without sarcoplasmic reticulum Ca2+ overload leading to reversal of the HF phenotype. Further, we demonstrate that the beneficial effects seen with GRK2 inhibition in HF were associated with marked amelioration of cardiac myocyte contractility and significant improvements in intracellular Ca2+ cycling effected by modulation of the cardiac L-type Ca2+ channels. A clearly novel finding is the compartmentalization of intracellular signaling by GRK2 and α2AR responsiveness in normal and failing cardiomyocytes through g(βγ)-mediated L-type calcium current disinhibition. Overall, our current results explain the beneficial effects of GRK2 knockout in cardiac myocytes with intracellular changes in Ca2+ cycling being orchestrated to improve overall cardiac-myocyte contractility. Furthermore, our study demonstrates that this interplay between GRK2 and the L-type Ca2+ channels might represent an attractive target to correct deranged Ca2+ cycling in the failing heart. Future gene therapies or pharmacological strategies will thus potentially directly target this interplay and might thus contribute to further improvements in clinical HF treatment.
Cardiac G-Protein–Coupled Receptor Kinase 2 Ablation Induces a Novel Ca\textsuperscript{2+} Handling Phenotype Resistant to Adverse Alterations and Remodeling After Myocardial Infarction

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Experimental animals

Conditional mice bearing floxed GRK2 alleles were described previously\(^1\), \(^2\). GRK2KO (αMHC-Cre x GRK2fl/fl) and WT (GRK2fl/fl) mice were maintained on a C57BL6 genetic background. All animal procedures and experiments were performed in accordance with the guidelines of the IACUC of Thomas Jefferson University.

Experimental protocols

GRK2KO and WT mice were 8-10 weeks of age when entering the current study. Unstressed normal mice and mice with coronary artery ligation (myocardial infarction (MI)) or sham-operation were studied. MI was induced by ligation of the LAD 2-3 mm below its origin as described previously \(^2\), \(^3\) and animals were studied 28 days post-MI. Sham animals underwent operation without ligation of the LAD. For the verapamil study mice were treated with verapamil starting 14 days post MI or sham operation until the end of the study period (42 days post MI). Verapamil treated mice received oral supplementation of verapamil (Sigma-Aldrich, St. Louis, Missouri, USA) in their drinking water. Verapamil was dissolved in 10% dextrose solution at a concentration of 1 mg/ml as described previously \(^4\).

Isolation of cardiac myocytes and maintenance of primary cultures

Adult mouse cardiac myocytes were isolated as previously described\(^5\). For Ca\(^{2+}\) transients and single myocyte contractility measurements, cells were used within 8 hours after isolation. For in vitro cell culture assays, freshly isolated cells were washed and resuspended with α-MEM (Gibco, Invitrogen Corporation, Carlsbad, California, USA) supplemented with Hanks’ salt, and then plated onto laminin (Invitrogen Corporation, Carlsbad, California, USA) pre-coated culture dishes. Cells were maintained in α-MEM with Hank’s salt solution supplemented with L-Glutamine (10 mmol/l), 2, 3-Butandionemonoxime (BDM, 10mmol/l)
(Sigma-Aldrich, St. Louis, Missouri, USA) and Insulin-Transferrin-Selenium (1:1000 dilution) (Gibco, Invitrogen Corporation, Carlsbad, California, USA) with a humidified atmosphere containing 2% CO₂ for 24 hours.

For each experiment involving isolated cells cardiac myocytes from one GRK2KO and one WT mouse were isolated at the same time and analyzed in parallel. The numbers are mentioned in each figure legend; an equal number of cells per animal were analysed from an equal number of animals per group.

**Ca²⁺ Transients and single myocyte contractility studies**

Freshly isolated cells were kept in BDM-free tyrodes solution containing 1mM CaCl₂. The cells were loaded for 10 min with Fura-2 (TefLabs, Austin, Texas, USA), thoroughly washed and placed on a laminin coated object slide. The cells were stimulated in an electrical field and continuously perfused with tyrodes containing 1mM CaCl₂ without (baseline) and with 10⁻⁸ M Isoproterenol (Sigma-Aldrich, St. Louis, Missouri, USA). Ca²⁺ transients were determined by recording the Fura2-ratio (340/380nm) (Fluorescence and Contractility System, IonOptix, Milton, Massachusetts, USA). Single-cell contractions were measured by video edge detection (Fluorescence and Contractility System, IonOptix, Milton, Massachusetts, USA).

**Measurement of SR Ca²⁺ load**

Myocytes were placed in a chamber mounted on an inverted Nikon microscope and perfused with normal physiological solution (Tyrode) containing 1mM Ca²⁺. Since MI may cause the increase of diastolic intracellular Ca²⁺, Indo-1 AM was used to measure Ca²⁺ transients and caffeine-induced Ca²⁺ transients for myocytes isolated from sham and post-MI hearts as described previously ⁶⁻⁸. To assess SR Ca²⁺ content, 10mM caffeine (Sigma-Aldrich, St. Louis, Missouri, USA) was applied on cells for 10 seconds after 4 field stimulations to reach steady state. The peak of caffeine-induced Ca²⁺ transient was used as the index of SR content. Peak Ca²⁺ concentrations of Ca²⁺ transients induced by field stimulation and caffeine
spritz were calculated as follows: $[\text{Ca}^{2+}] = K_d \times R / ((K_d/\text{[Ca}^{2+}\text{]}_{\text{rest}})+1-R)$, where $R$ is the ratio of emitted fluorescence to the resting emitting fluorescence. $\text{[Ca}^{2+}\text{]}_{\text{rest}}$ was considered as 100nM and $K_d$ was 1100nM. The ratio of peak $\text{Ca}^{2+}$ concentration induced by stimulation to peak $\text{Ca}^{2+}$ concentration induced by caffeine was used as the index of fractional release.

**Electrophysiology**

Whole cell L-type $\text{Ca}^{2+}$ channel current ($I_{\text{Ca,L}}$) was measured in Na$^+$- and K$^+$-free solutions at 37°C using techniques described in detail previously.

Single L-type Ca2+ channel currents were recorded in cell-attached patches with an Axopatch 200B amplifier and Clampex 10. The holding potential was -90mV assuming the intracellular resting membrane potential was about -70mV (the averaged resting membrane potentials in control and DTG myocytes during electrophysiological measurements) and depolarized to 10mV. The recording glass pipette contained (in mM): BaCl$_2$, 70; Sucrose, 70; NMDG, 10; HEPES, 10; TEA-Cl (tetraammonium chloride), 10; tetrodotoxin (TTX), 0.05; and 4-Aminopyridine (4-AP), 5; pH7.4 with TEA-OH. The bath solution was normal Tyrode solution containing (in mM): NaCl 150, KCl 5.4, MgCl$_2$ 1.2, HEPES 5, Glucose 10, Na-pyruvate 2, CaCl$_2$ 1, pH to 7.4 with NaOH. After 400 continuous sweeps of recording at 0.5Hz, the external solution was changed to a H89 (5µM) containing bath solution and 15 minutes were waited to allow H89 to fully take effect. Then, another 400 continuous sweeps of recording were made. Single-channel and whole-cell $\text{Ca}^{2+}$ currents were analyzed with Clampfit 10. To quantify the amount of LTCCs on the surface membrane of the myocytes, charge movement measurements of the LTCC were done with pCLAMP10 and an Axon 200B amplifier as described previously$^{10}$. Only myocytes with minimal (<10%) rundown of $I_{\text{Ca,L}}$ were included in the data sets. $I_{\text{Ca,L}}$ was measured at baseline and under stimulation with $10^{-6}$M Isoproterenol. To study the effect of H89 (a PKA inhibitor, Sigma-Aldrich, 5µM) on $I_{\text{Ca,L}}$, 5µM H89 was included with the pipette solution for 10 minutes to allow adequate diffusion of H89 into the cell to inhibit PKA.
Sodium-calcium exchange current (INCX) was recorded as described previously\textsuperscript{11}. The myocyte was bathed in a K\textsuperscript{+}-free solution containing (in mmol/L): NaCl 145, MgCl\textsubscript{2} 1, HEPES 5, CaCl\textsubscript{2} 2, CsCl 5, glucose 10, ouabain 0.02, nifedipine, 0.01, pH 7.4 adjusted with NaOH. The internal solution contained (in mM): CsCl 65, NaCl 20, Na\textsubscript{2}ATP 5, CaCl\textsubscript{2} 6, MgCl\textsubscript{2} 4, HEPES 10, TEA-Cl 20, EGTA 21, ryanodine 0.0005, pH 7.2 with CsOH. The cell was dialyzed for 10 minutes after rupturing the patch and the membrane current was recorded with a ramp test (+80mV to -80mV at 100mV/s) following a 100-ms depolarization to +80 from the holding potential of -40mV. During the recording, the bath solution then was changed to the K\textsuperscript{+}-free solution with 5mmol/L Ni\textsuperscript{2+}. Recording was stopped once a stable effect of Ni\textsuperscript{2+} was seen and the Ni\textsuperscript{2+}-sensitive current was INCX.

RNA isolation, reverse transcription and quantitative real-time PCR
RNA was isolated from snap-frozen samples of the remote zone collected at 28 days post MI or sham operation or from cardiac myocytes isolated at 42 days post MI or sham operation (verapamil study) as described previously \textsuperscript{2}. cDNA was synthesized and expression levels of NCX and BNP were analyzed using quantitative Real-Time PCR\textsuperscript{2}; 28S or 18S mRNA levels were used for normalization.

Western blot analysis
Western blotting was performed as described previously \textsuperscript{3}. Cardiac protein levels of GRK2 (sc-562, C-15, Santa Cruz Biotechnology, 1:5,000), SERCA (Clone II-H-11, Sigma-Aldrich, St. Louis, Missouri, USA), PLB, pPLB S16, pPLB Thr17 (A010-14, -12, and -13, Badrilla, Leeds, United Kingdom), and GAPDH (clone 6C5, Millipore, Billerica, Massachusetts, USA) were assessed in cardiac myocyte cellular preparations. Protein content was quantified with the BioRad DC Protein Assay (BioRad Laboratories, Richmond, California, USA). Protein samples were separated by 4-20% SDS-PAGE (Invitrogen Corporation, Carlsbad, California, USA), and proteins were transferred to PVDF membrane (Millipore Corporation, Billerica, Massachusetts, USA) and probed with the first antibody at 4°C overnight. The proteins were
stained with a corresponding Alexa Fluor 680- (Molecular Probes; 1:10.000) or IRDye 800CW-coupled (Rockland Inc.; 1:10.000) secondary antibody, followed by visualization of the proteins with a LI-COR infrared imager (Odyssey, LI-COR, Lincoln, Nebraska, USA), and quantitative densitometric analysis was performed applying Odyssey version 2.0 infrared imaging software. α1c immunoprecipitation was performed with the Dynabeads Protein A for Immunoprecipitation Kit (Invitrogen Corporation, Carlsbad, California, USA) with 700µg crude protein extracted from each frozen heart according to the manufacture’s instruction. The antibody against α1c for immunoprecipitation was purchased from Millipore (Billerica, MA, USA) and the antibodies for the detection of α1c and p1928 α1c were from NeuroMab (Davis, CA, USA).

**Determination of PKA/PDE4 dependent hypophosphorylation of PLB following loss of GRK2**

A PDE 4 specific inhibitor, rolipram (10mg/kg BW, i.p.) (Sigma-Aldrich, St. Louis, Missouri, USA) was injected into WT and GRK2KO mice for 2 hours to allow it to take effect. Then the hearts were snap-frozen in liquid nitrogen. Western blotting against PLB and pPLB S16 (A010-14, and -12, Badrilla, Leeds, United Kingdom) and GAPDH (clone 6C5, Millipore, Billerica, Massachusetts, USA) was performed.

**Echocardiographic analysis of cardiac function**

Two-dimensional transthoracic echocardiography was acquired with a 12-MHz probe and a Vevo770 imaging system (VisualSonics Inc., Toronto, Ontario, Canada) both in sham and infarcted mice as described in details elsewhere. LV diameters and contractility (fractional shortening, FS% = ([LVEDD -LVESD]/LVEDD) × 100) were assessed by an M-Mode recording in the parasternal short axis view.
**Statistical analysis**

Data are expressed as mean±SEM. An unpaired two tailed t-test or a one-way ANOVA and a two-way ANOVA (linear mixed effects model) were performed with SAS 9.3 for between-group comparisons followed by a post-hoc Bonferroni adjustment. For all tests, a P value < 0.05 was considered significant.

**SUPPLEMENTAL RESULTS**

*Loss of GRK2 in cardiac myocytes before MI reduced the extent of cardiac dysfunction.* To study the consequence of GRK2 loss on the development of HF, GRK2KO mice and their corresponding littermate controls (WT) were subjected to MI. Infarct size was not different at 24h post MI in GRK2KO mice as compared to WT mice (data not shown). *In vivo* cardiac function was assessed by echocardiography twenty-eight days after MI. In sham operated animals no differences were observed between both groups. As we have recently shown post-MI cardiac function and ventricular remodeling were significantly improved after the loss of myocyte GRK2 despite deterioration of cardiac function in corresponding WT mice (see online data supplement, Table 1).

**SUPPLEMENTAL TABLES**

|                          | Sham WT | Sham GRK2KO | MI WT | MI GRK2KO |
|--------------------------|---------|-------------|-------|-----------|
| Heart Rate [beats/min]   | 471 ± 22| 452 ± 14    | 415 ± 9| 433 ± 12 |
| End-Diastolic Diameter [mm] | 3.08 ± 0.12 | 3.14 ± 0.16 | 4.44 ± 0.13 | 4.03 ± 0.09 * |
| End-Systolic Diameter [mm] | 1.81 ± 0.09 | 1.87 ± 0.12 | 3.63 ± 0.13 | 3.03 ± 0.09 * |
| Fractional Shortening [%] | 41.41 ± 1.53 | 40.60 ± 1.03 | 18.21 ± 0.94 | 24.88 ± 1.34 * |
Data supplement table 1: Echocardiography for determination of cardiac function in WT and GRK2KO mice with (MI) or without MI (Sham). In vivo cardiac function was assessed by echocardiography twenty-eight days after MI. n=10 animals/sham group, n=15-19 animals/MI group, *: p<0.05.

|                           | Sham WT   | Sham GRK2KO | MI WT     | MI GRK2KO |
|---------------------------|-----------|-------------|-----------|-----------|
| FS at 0.5Hz [%]           | 6.24±0.42 | 7.16±0.44   | 2.56±0.19  | 4.19±0.14  |
| [Ca2+] peak at 0.5Hz [Fura-2,340/380] | 0.09±0.005 | 0.10±0.004 | 0.07±0.002 | 0.108±0.003 |
| FS at 2Hz [%]             | 4.9±0.51  | 6.4±0.58    | 3.5±0.48   | 5.6±0.35   |
| [Ca2+] peak at 2Hz [Fura-2, 340/380] | 0.07±0.003 | 0.08±0.004 | 0.04±0.006 | 0.08±0.005 |
| SR Ca2+ load [indo-1, 410/480] | 0.2±0.02  | 0.22±0.01   | 0.15±0.01  | 0.26±0.04  |
| Fractional Ca2+ release [%]| 64±1.3    | 75.4±4.4    | 70.6±5.1   | 83.9±5.9   |
| lCa,L peak [pA/pF]        | -10.8±2.0 | -18.57±0.9  | -3.5±1.3   | -5.0±1.6   |

Data supplement table 2: Myocyte properties in WT and GRK2KO myocytes without or with MI. Averaged myocyte fractional shortening at 0.5 Hz (FS at 0.5Hz [%]) and 2.0 Hz (FS at 2Hz [%]) stimulation frequencies under basal conditions; averaged Fura-2 ratio amplitude at 0.5 Hz ([Ca2+] peak at 0.5Hz [Fura-2,340/380]) and 2.0 Hz ([Ca2+] peak at 2Hz [Fura-2,340/380]) stimulation frequencies under basal conditions; for these measurements a total of 48-63 cardiac myocytes from 3-6 different hearts were measured for each group. Caffeine-induced peak intracellular Ca2+ amplitudes under baseline conditions (SR Ca2+ load [indo-1, 410/480]); fractional release calculated as the ratio of peak Ca2+ concentration induced by field stimulation to peak Ca2+ concentration induced by caffeine (Fractional Ca2+ release [%]); for these measurements a total of 14-29 cardiac myocytes from 3-5 different hearts were analyzed per group. Peak lCa,L (ICa-L peak [pA/pF]) under basal conditions; 3-5 hearts were analyzed for each group. One-way ANOVA. *: p<0.05: GRK2KO sham vs. WT sham, #: p<0.05, WT MI vs. WT sham, %: p<0.05, GRK2KO MI vs. WT MI.
SUPPLEMENTAL REFERENCES

1. Matkovich SJ, Diwan A, Klanke JL, Hammer DJ, Marreez Y, Odley AM, Brunskill EW, Koch WJ, Schwartz RJ, Dorn GW, 2nd. Cardiac-specific ablation of g-protein receptor kinase 2 redefines its roles in heart development and beta-adrenergic signaling. Circ Res. 2006;99:996-1003

2. Raake PW, Vinge LE, Gao E, Boucher M, Rengo G, Chen X, DeGeorge BR, Jr., Matkovich S, Houser SR, Most P, Eckhart AD, Dorn GW, 2nd, Koch WJ. G protein-coupled receptor kinase 2 ablation in cardiac myocytes before or after myocardial infarction prevents heart failure. Circ Res. 2008;103:413-422

3. Most P, Seifert H, Gao E, Funakoshi H, Volkers M, Heierhorst J, Remppis A, Pleger ST, DeGeorge BR, Jr., Eckhart AD, Feldman AM, Koch WJ. Cardiac s100a1 protein levels determine contractile performance and propensity toward heart failure after myocardial infarction. Circulation. 2006;114:1258-1268

4. Cohn RD, Durbeej M, Moore SA, Coral-Vazquez R, Prouty S, Campbell KP. Prevention of cardiomyopathy in mouse models lacking the smooth muscle sarcoglycan-sarcospan complex. J Clin Invest. 2001;107:R1-7

5. Zhou YY, Wang SQ, Zhu WZ, Chruscinski A, Kobilka BK, Ziman B, Wang S, Lakatta EG, Cheng H, Xiao RP. Culture and adenoviral infection of adult mouse cardiac myocytes: Methods for cellular genetic physiology. Am J Physiol Heart Circ Physiol. 2000;279:H429-436

6. Tocchetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Di Benedetto G, O'Rourke B, Gao WD, Wink DA, Toscano JP, Zaccolo M, Bers DM, Valdivia HH, Cheng H, Kass DA, Paolocci N. Nitroxyl improves cellular heart function by directly enhancing cardiac sarcolemmal Ca2+ cycling. Circ Res. 2007;100:96-104

7. Wei SK, Ruknudin A, Hanlon SU, McCurley JM, Schulze DH, Haigney MC. Protein kinase a hyperphosphorylation increases basal current but decreases beta-adrenergic responsiveness of the sarcosomal Na+/Ca2+ exchanger in failing pig myocytes. Circulation research. 2003;92:897-903

8. Terracciano CM, Souza AI, Philipson KD, MacLeod KT. Na+-Ca2+ exchange and sarcoplasmic reticular calcium Ca2+ regulation in ventricular myocytes from transgenic mice overexpressing the Na+/Ca2+ exchanger. J Physiol. 1998;512 (Pt 3):651-667

9. Ginsburg KS, Bers DM. Modulation of excitation-contraction coupling by isoproterenol in cardiomyocytes with controlled SR Ca2+ load and Ca2+ current trigger. J Physiol. 2004;556:463-480

10. Chen X, Placentin V, 3rd, Furukawa S, Goldman B, Margulies KB, Houser SR. L-type Ca2+ channel density and regulation are altered in failing human ventricular myocytes and recover after support with mechanical assist devices. Circ Res. 2002;91:517-524

11. Nakayama H, Chen X, Baines CP, Klevitsky R, Zhang X, Zhang H, Jaleel N, Chua BH, Hewett TE, Robbins J, Houser SR, Molkentin JD. Ca2+- and mitochondrial-dependent cardiomyocyte necrosis as a primary mediator of heart failure. J Clin Invest. 2007;117:2431-2444
심장에서 GRK2 효소 제거는 세포 내 칼슘 이온 처리를 원활히 하여 심근경색 후 심장재형성의 악화를 막을 수 있다

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Summary

배경
GRK2(G-protein-coupled receptor kinase 2)는 심장에서 베타 아드레너직(β-adrenergic) 수용체와 그 신호 전달체계를 조절하는 가장 중요한 단백 효소이다. 심장에서 GRK2를 제거하면 심부전 진행을 방해하는데, 그 세포학적 기전에 대해서는 아직 잘 알려져 있지 않아 본 연구를 통하여 알아보기로 한다.

방법 및 결과
일반 생쥐와 GRK2 유전자를 제거한 생쥐(GRK2KO)를 각각 겉보기(sham) 수술 혹은 심근경색(myocardial infarction) 유발 수술 후 심근세포를 분리하여, 수축력과 칼슘 이온 처리능(Ca²⁺ handling)을 평가하였다. 겉보기 수술을 한 경우 일반 생쥐와 GRK2KO 모두에서 심근 수축력과 칼슘 이온 이동 양상은 유사하였으나, GRK2KO에서 SR(sarcoplasmic reticulum)에 저장된 칼슘 이온이 더 낮게 관찰되었다. sodium-Ca²⁺ exchanger(NCX)의 활성도는 증가되고, SR Ca²⁺ ATPase(SERCA)는 감소되어 있었으며, 이는 protein kinase A에 의한 phosphodiesterase 4의 활성화가 phospholamban(PLB)을 인산화하여 활성도를 증가시키기 때문이다. 이러한 칼슘 이온의 세포 내 처리 변화는 L-type Ca²⁺ channel(LTCC) current를 증가시키며 SR Ca²⁺ 분비의 분획이 증가하는 것으로 알 수 있다. 베타 아드레너직 수용체 자극(isoproterenol 투여)은 GRK2KO의 심근 수축력과 Ca²⁺ 이동을 현저히 증가시키는데, 이는 LTCC와는 관계없이 SR Ca²⁺의 증가가 원인이다. 흥미로운 것은 심근경색 후 GRK2KO 생쥐가 일반 생쥐에 비하여 더 좋은 심장 기능을 유지하는데, 이는 더 개선된 칼슘 이온의 처리 표현형을 갖기 때문이다. 또한, 심근경색 후 SR 내의 칼슘 이온은 일반 생쥐보다 GRK2KO 생쥐에서 더욱 잘 유지되는데, 이는 GRK2KO 생쥐에서는 LTCC current 농도가 더 잘 유지되고 NCX의 증가가 없기 때문이다. 이런 GRK2KO 생쥐의 이로운 현상은 LTCC 차단제인 verapamil을 투여하면 사라진다.

결론
본 연구는 GRK2에 의한 LTCC current와 SR 칼슘 이온에 대한 차별화된 새로운 기전을 밝혔다. 또한, GRK2를 심장으로부터 제거함으로써 심근경색 후 심장재형성을 억제할 수 있는 새로운 칼슘 이온 처리 모델을 시사했다.
심근 수축력 변화와 칼슘 이온의 순환은 심부전 발생 과 진행에 중심적인 역할을 한다. 심부전이 진행되는 심장에서는 sarcoplasmic reticulum 내에 저장된 SR Ca\(^{2+}\) 양이 감소하며, Ca\(^{2+}\) 이동이 감소하고 연장되어, 결국 심근 수축력을 감소시킨다. 주로 심부전에서 이러한 현상은 NCX의 활성도 증가, SERCA 발현 및 활성도 감소(이는 PLB 인산화 감소에 의함), PLB/SERCA 비율 증가 및 LTCC 변화 등으로 알려져 있다. 심부전 치료를 위한 단으로 이양의 칼슘 이온 처리나 excitation-contraction coupling을 조정하면 가능할 것으로 가정하고 많은 연구가 수행되었다.

심부전에서 교감신경계의 흥분은 심근 수축력을 증가시키고, 칼슘 이온의 순환을 촉진하여 심장기능을 향상시킨다. 급성 심부전에서 이러한 현상은 전신순환과 주요 장기 혈액 관류를 개선시켜 임상적으로 호전되었다. 그러나 만성적 교감신경계의 활성화는 GRK2 활성화와 함께 베타 아드레날린 수용체의 이상태나 기능적 저하를 동반하여 나쁜 장기적 예후를 보인다. 심부전 치료의 가능성은 이런 심장 교감신경계를 조절함으로써 가능하다는 것이 알려져 있다. GRK2의 활성도 억제는 c-terminal domain 물질인 BARKc를 과발현시킴으로써 심부전 진행을 억제할 수 있는 것으로 보고되고 있는데, 이는 심부전 환자에서 베타차단제가 심부전 진행을 막는 것과 유사한 기전을 일부 가진다. 그러나 GRK2 억제가 심부전 진행을 막는 기전에 대해서는 주로 Gs, G-protein 신호 전달체계나 β-arrestin 신호 등에 초점을 맞춰왔다.

본 논문의 새로운 점은 위에 나열한 심부전 관련 칼슘 이온 처리 기전에 덧붙여 GRK2가 칼슘 이온 순환과 심근 수축 두 위치에서 작용한다는 새로운 사실을 밝혔다. 하나는 세포 내의 sarcoplasmic reticulum의 칼슘 이온 이동과 저장에 관여하며, 다른 하나는 세포막 (sarcolemma)의 LTCC에 작용한다는 것이다. 더 나아가 심장의 GRK2 효소를 제거시켜 심근 수축력의 항상과 칼슘 이온 순환의 호전을 가져오는데, 이는 GRK2가 LTCC에 대한 작용을 억제함으로써 나타나게 된 결과이다. 본 논문은 향후 심부전 환자의 칼슘 이온 순환의 개선과 예후를 향상시키기 위한 치료의 목표로서 GRK2와 LTCC의 역할에 대한 중요한 한 가지의 제시가 될 수 있겠다.

References
1. Rengo G, Lympersopoulos A, Zincarelli C, Femminella G, Liccardo D, Pagano G, de Lucia C, Cannavo A, Gargiulo P, Ferrara N, Perrone Filardi P, Koch W, Leosco D. Blockade of β-adrenoceptors restores the GRK2-mediated adrenal α(2)-adrenoceptor-catecholamine production axis in heart failure. Br J Pharmacol. 2012;166:2430-2440.
2. Brinks H, Das A, Koch WJ. A role for GRK2 in myocardial ischemic injury: indicators of a potential future therapy and diagnostic. Future Cardiol. 2011;7:547-556.
3. Santulli G, Campanile A, Spinelli L, Assante di Panzillo E, Ciccarelli M, Trimarco B, Iaccarino G. G protein-coupled receptor kinase 2 in patients with acute myocardial infarction. Am J Cardiol. 2011;107:1125-1130.
4. Brinks H, Das A, Koch WJ. A role for GRK2 in myocardial ischemic injury: indicators of a potential future therapy and diagnostic. Future Cardiol. 2011;7:547-556.
5. Brinks H, Boucher M, Guo E, Chuprun JK, Pesant S, Raaka PW, Huang ZM, Wang X, Qi G, Gumpert A, Harris DM, Eckhart AD, Most P, Koch WJ. Level of G protein-coupled receptor kinase-2 determines myocardial ischemia/reperfusion injury via pro- and anti-apoptotic mechanisms. Circ Res. 2010;107:1140-1149.
Cardiac G-Protein–Coupled Receptor Kinase 2 Ablation Induces a Novel Ca\(^{2+}\) Handling Phenotype Resistant to Adverse Alterations and Remodeling After Myocardial Infarction

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**Background**—G-protein–coupled receptor kinase 2 (GRK2) is a primary regulator of β-adrenergic signaling in the heart. G-protein–coupled receptor kinase 2 ablation impedes heart failure development, but elucidation of the cellular mechanisms has not been achieved, and such elucidation is the aim of this study.

**Methods and Results**—Myocyte contractility, Ca\(^{2+}\) handling and excitation-contraction coupling were studied in isolated cardiomyocytes from wild-type and GRK2 knockout (GRK2KO) mice without (sham) or with myocardial infarction (MI). In cardiac myocytes isolated from unstressed wild-type and GRK2KO hearts, myocyte contractions and Ca\(^{2+}\) transients were similar, but GRK2KO myocytes had lower sarcoplasmic reticulum (SR) Ca\(^{2+}\) content because of increased sodium-Ca\(^{2+}\) exchanger activity and inhibited SR Ca\(^{2+}\) ATPase by local protein kinase A–mediated activation of phosphodiesterase 4 resulting in hypophosphorylated phospholamban. This Ca\(^{2+}\) handling phenotype is explained by a higher fractional SR Ca\(^{2+}\) release induced by increased L-type Ca\(^{2+}\) channel currents. After β-adrenergic stimulation, GRK2KO myocytes revealed significant increases in contractility and Ca\(^{2+}\) transients, which were not mediated through cardiac L-type Ca\(^{2+}\) channels but through an increased SR Ca\(^{2+}\). Interestingly, post-MI GRK2KO mice showed better cardiac function than post-MI control mice, which is explained by an improved Ca\(^{2+}\) handling phenotype. The SR Ca\(^{2+}\) content was better maintained in post-MI GRK2KO myocytes than in post-MI control myocytes because of better-maintained L-type Ca\(^{2+}\) channel current density and no increase in sodium-Ca\(^{2+}\) exchanger in GRK2KO myocytes. An L-type Ca\(^{2+}\) channel blocker, verapamil, reversed some beneficial effects of GRK2KO.

**Conclusions**—These data argue for novel differential regulation of L-type Ca\(^{2+}\) channel currents and SR load by GRK2. G-protein–coupled receptor kinase 2 ablation represents a novel beneficial Ca\(^{2+}\) handling phenotype resisting adverse remodeling after MI. ([Circulation. 2012;125:2108-2118.])

**Key Words:** calcium ■ experimental models ■ heart failure ■ excitation contraction coupling ■ G-Protein coupled receptor kinase 2

Alterations in myocyte contractility and Ca\(^{2+}\) cycling are hallmarks of the progression of heart failure (HF). In failing cardiac myocytes, the sarcoplasmic reticulum (SR) Ca\(^{2+}\) content is decreased and the Ca\(^{2+}\) transient is decreased and prolonged, resulting in depressed myocyte contractility. This is generally considered to be a consequence of increased sodium-Ca\(^{2+}\) exchanger (NCX) activity, reduced SR Ca\(^{2+}\) ATPase (SERCA) expression and activity (probably due to decreased phosphorylation [PLB] phosphorylation), and increased PLB/SERCA ATPase ratio, as well as an augmented...
The increased activity of the sympathetic nervous system associated with HF is a compensation to normalize cardiac function by enhancing Ca\(^{2+}\) cycling and maximize contractile force through the \(\beta\)-adrenergic signaling pathway. In acute HF, these changes can improve systemic perfusion whereas in chronic HF the augmentation in catecholamines is associated with mortality\(^6\) and results in a downregulation of \(\beta\)-adrenergic receptors (\(\beta\)ARs) promoted by upregulated G protein-coupled receptor kinase 2 (GRK2). G protein-coupled receptor kinase 2 is the primary GRK in the heart and a prototype regulator of \(\beta\)AR signaling.\(^7\) We have previously identified GRK2 as a culprit in the progression of HF, and GRK2 inhibition (by expression of its c-terminal domain, called \(\beta\)ARKct) or gene silencing has rescued disparate models of HF.\(^8\)-\(^10\) Our recent study indicates that the benefits of \(\beta\)ARKct could be related to enhanced myocyte contractility by increasing LTCC currents and its responsiveness to \(\beta\)-adrenergic agonists.\(^1\)\(^1\) However, the exact underlying cellular mechanisms for these beneficial effects in HF after \(\beta\)ARKct expression or GRK2 ablation are not clearly defined. It is especially important to define these mechanisms because in light of the recent success of \(\beta\)AR blocker therapy in clinical HF management, the results with \(\beta\)ARKct and GRK2 silencing appear paradoxical, as the major function of GRK2 in cardiac myocytes is to dampen \(\beta\)AR signaling in a manner similar to that of \(\beta\)AR blockade. We have found that \(\beta\)ARKct expression can cause a molecular remodeling of the cardiac \(\beta\)AR system with receptor upregulation and improved \(\beta\)AR signaling, and a recent study with chronic mediated \(\beta\)ARKct expression in a rat HF model showed that myocardial \(\beta\)AR changes are probably down-stream of neurohormonal lowering including reduction in sympathetic nervous system activity.\(^12\) In this regard, the role of GRK2 inhibition must mechanistically go beyond resensitizing \(\beta\)ARs and fully understanding GRK2-dependent signaling pathways might enlighten novel therapeutic targets.

To date, little is known about how GRK2 specifically alters cardiac myocyte function and Ca\(^{2+}\) cycling in normal and failing cardiac myocytes. The present study was designed to define the role of GRK2 and GRK2-dependent signaling in excitation-contraction coupling (ECC) in normal and diseased hearts. We used our previously characterized cardiac-specific GRK2KO mice\(^\text{10,13}\) to study myocyte ECC coupling and Ca\(^{2+}\) homeostasis in cardiac myocytes from mice with or without post-MI ischemic HF. We demonstrate for the first time that loss of GRK2 induces a distinct Ca\(^{2+}\) handling phenotype: Myocyte contractility and Ca\(^{2+}\) handling are normal even though the SR Ca\(^{2+}\) content is reduced because there is an increase in LTCC activities and resulting increases in LTCC currents (\(I_{Ca,L}\)) with a compensatory increase in NCX activity. This Ca\(^{2+}\) handling phenotype brought about by GRK2 ablation is resistant to adverse Ca\(^{2+}\) handling remodeling after MI and leads to better cardiac function in GRK2KO mice post MI.

Methods

Conditional mice bearing floxed GRK2 alleles were described previously.\(^\text{10,13}\) G-protein-coupled receptor kinase 2 KO (\(\alpha\) myosin heavy chain – Cre-recombinase \(\times\) GRK2flox/flox) and wild-type (WT) (GRK2flox/flox) mice were maintained on a C57BL/6 genetic background. All animal procedures and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Thomas Jefferson University. G-protein-coupled receptor kinase 2 KO and WT mice were 8 to 10 weeks of age when entering the study. Unstressed normal mice and mice with coronary artery ligation (myocardial infarction [MI]) or sham operation were studied. Myocardial infarction was induced by ligating the left anterior descending coronary artery at 2 to 3 mm below its origin as described previously.\(^\text{13,14}\) and animals were studied 28 days post-MI or sham operation until the end of the study period (42 days after MI). Verapamil-treated mice received oral supplementation of verapamil (Sigma-Aldrich, St. Louis, MO) as described previously\(^\text{13}\) and were evaluated by echocardiography. Cardiac myocytes were isolated cultured from animals. Myocyte Ca\(^{2+}\) transients and contractions, SR Ca\(^{2+}\) load, \(I_{Ca,L}\), and NCX-Activity INCX were measured. Quantitative real-time polymerase chain reaction and Western blot analysis were performed for gene-expression assessment. Detailed description of experimental procedures is available in the online-only Data Supplement.

Results

Myocyte GRK2 Ablation Enhances Adrenergic Responsiveness of Cellular Contractility and Ca\(^{2+}\) Transients

Our previous study has shown that GRK2 KO mice have cardiac function comparable to control mice at baseline (online-only Data Supplement Table I), but their cardiac function responds better to \(\beta\)-adrenergic stimulation.\(^\text{10}\) Here, we determine the cellular mechanisms for this observation. Myocyte contraction and intracellular Ca\(^{2+}\) transients were recorded from WT and GRK2KO myocytes under baseline conditions and after \(\beta\)AR stimulation. Myocytes from both lines revealed a similar fractional shortening when paced at both 0.5Hz and 2Hz (Figure 1A–1C). However, after isoproterenol (ISO), myocytes from GRK2KO mice showed a significantly greater increase in fractional shortening (Figure 1A–1C and online-only Data Supplement Table II). In a good agreement, as shown in Figure 1D, at baseline, the characteristics and amplitude of the Ca\(^{2+}\) transients (as \(\Delta\)Fura-2 340/380 nm ratio) were similar in both groups of myocytes. Stimulation with ISO mediated an anticipated increase in the amplitude of the Fura-2 ratio in WT cardiac myocytes and a greater increase in GRK2KO cardiac myocytes (Figure 1D–1F, online-only Data Supplement Table II). We observed no differences in the Ca\(^{2+}\) transient decay time constants at baseline and after ISO between groups.
GRK2 Silencing in Myocytes Enhances ECC Efficiency

Myocyte contractility and Ca\(^{2+}\) transients are determined by Ca\(^{2+}\) release from the SR.\(^{16}\) We measured SR Ca\(^{2+}\) content by rapid application of caffeine (caffeine spritz) at baseline or after ISO stimulation. Figure 2A shows representative tracings of cytosolic Ca\(^{2+}\) transients (measured with indo-1 AM) induced by caffeine spritz after 4 field stimulations in WT and GRK2KO cardiac myocytes to measure the SR Ca\(^{2+}\) load. At baseline, SR Ca\(^{2+}\) load was less in GRK2 myocytes than in control myocytes (Figure 2B); in response to ISO, the SR Ca\(^{2+}\) load in cardiac myocytes from both WT and GRK2KO mice was significantly increased, but GRK2KO myocytes had a greater increase (Figure 2B). Interestingly, a significantly higher fractional release of Ca\(^{2+}\) was observed in GRK2KO cardiac myocytes compared with WT myocytes at both baseline and after ISO (Figure 2C and online-only Data Supplement Table II).
Loss of Myocyte GRK2 Enhances I_{Ca,L} by a Local Protein Kinase A–Dependent Mechanism but Blunts Its βAR Responsiveness

To explore the underlying cellular mechanisms for the reduced SR Ca^{2+} content with enhanced EC coupling efficiency in GRK2 KO myocytes, we measured the I_{Ca,L} in GRK2KO and control myocytes because I_{Ca,L} serves as both the trigger of Ca^{2+} release from the SR and the source for loading the SR. Peak I_{Ca,L} density at baseline was significantly greater in GRK2KO compared with WT cardiac myocytes (Figure 3A and 3B and online-only Data Supplement Table II). When stimulated with a saturating dose of ISO (10^{-6} M), peak I_{Ca,L} was increased to the same level in cardiac myocytes from both mouse lines (Figure 3A and 3B), suggesting that the LTCC density was similar in both groups. The voltage dependency of channel activation was shifted to more negative voltages in GRK2KO myocytes (Figure 3A and 3B and online-only Data Supplement Table II). When stimulated with a saturating dose of ISO (10^{-6} M), peak I_{Ca,L} was increased to the same level in cardiac myocytes from both mouse lines (Figure 3A and 3B), suggesting that the LTCC density was similar in both groups. The voltage dependency of channel activation was shifted to more negative voltages in GRK2KO myocytes (Figure 3A and 3B and online-only Data Supplement Table II). When stimulated with a saturating dose of ISO (10^{-6} M), peak I_{Ca,L} was increased to the same level in cardiac myocytes from both mouse lines (Figure 3A and 3B), suggesting that the LTCC density was similar in both groups.

The availability (Figure 3K) and the open probability (Figure 3L and 3M) were not significantly different in GRK2KO and control myocytes because I_{Ca,L} serves as both the trigger of Ca^{2+} release from the SR and the source for loading the SR. When stimulated with a saturating dose of ISO (10^{-6} M), peak I_{Ca,L} was increased to the same level in cardiac myocytes from both mouse lines (Figure 3A and 3B), suggesting that the LTCC density was similar in both groups.

The single-channel study further confirmed that the LTCC in GRK2KO myocytes were in such a high activity state that it loses responsiveness to β-adrenergic stimulation. To explore the underlying mechanisms for these changes in I_{Ca,L} properties, we tested whether the increased I_{Ca,L} was due to increased available channels on the surface membrane, the increase of channel activities at single-channel levels, or both. Charge movement was used to quantify the number of available channels on the surface membrane of KO and control WT myocytes. Figure 3E shows that there was no significant difference in the charge movements of the LTCC induced by various depolarizing voltages, a result which indicates that there was no significant alteration of LTCC density on the membrane and that the increased whole-cell I_{Ca,L} could be due to the increased single-channel activities.

Immunoprecipitation of α1c, the pore-forming subunit of the LTCC, from the same amount of proteins in GRK2KO and control hearts showed no difference in α1c expression (Figure 3N). These data suggest that the LTCC in GRK2KO myocytes must have higher than normal activity, a result supported by single-channel recording of LTCC activities (Figure 3J–3M). The availability (Figure 3K) and the open probabilities (Figure 3L and 3M) were not significantly different in GRK2KO myocytes, a result that could fully explain the increase in whole-cell I_{Ca,L} in KO myocytes.

Enhanced phosphorylation of the LTCC by protein kinase A (PKA) may result in increased LTCC activity. Previously, we have shown that βARs carry constitutive activation to activate PKA locally to phosphorylate the LTCC. Here, we tested whether high LTCC activity was mediated by a PKA-dependent mechanism. H89, a PKA-specific inhibitor, was used, and it normalized the current density and voltage-dependent activation of the LTCC in KO myocytes but had no significant effect on LTCCs in WT myocytes (Figure 3F–3I). The single-channel study further confirmed that the heightened LTCC activity in the KO myocytes was due to PKA activation because H89 also normalized the increased channel activity in KO myocytes (Figure 3J–3M). The phosphorylation of α1c at Ser1928, a PKA site, was shown to be greater in GRK2KO hearts than in control hearts (Figure 3N and 3O).

Loss of GRK2 in Myocytes Increases Na^{+}/Ca^{2+}-Exchanger Expression

Because there is a decreased SR Ca^{2+} content with increased I_{Ca,L} in GRK2KO myocytes, there should be increased Ca^{2+} efflux out of GRK2KO myocytes. In ventricular myocytes, the major route of Ca^{2+} efflux is through the NCX. One way to measure NCX activity is to examine the decay rate of caffeine-induced Ca^{2+} transients that can be fit by a single exponential decay equation. The tau value was significantly smaller in the GRK2KO myocytes than in control myocytes, and ISO did not change these values (Figure 4A), suggesting that the NCX...
activity at baseline is increased in GRK2KO myocytes compared with WT myocytes, a result that was confirmed by direct measurement of NCX current (Figure 4B) and NCX protein expression (Figure 4C and 4D).

Loss of GRK2 From Myocytes Decreases Basal PLB Phosphorylation but Increases Its Responsiveness to \( \beta \)-AR Stimulation

The loading of the SR with \( \text{Ca}^{2+} \) depends on the competition between the extrusion of \( \text{Ca}^{2+} \) out of the cell (mainly through NCX) and the resequestration of \( \text{Ca}^{2+} \) into SR by SERCA, which is regulated by PLB. Dephosphorylated PLB exerts a tonic inhibition on SERCA activity. For these reasons, we determined the expression level of SERCA and PLB as well as the phosphorylation level of PLB. The expression of SERCA and PLB was not significantly altered by silencing GRK2 (Figure 4E–4H). However, the phosphorylation of PLB at Ser16, a PKA site, was significantly reduced in GRK2KO hearts but the phosphorylation of PLB at Thr17 site was not altered (Figure 4G and 4H). When myocytes were stimulated with the \( \beta \)-AR agonist ISO, a robust increase in the phosphorylation of PLB at Ser16 sites was observed in both WT and GRK2KO cardiac myocytes (Figure 4I and 4J). However, a significant leftward shift of the dose–response curve was found in GRK2KO myocytes compared with WT cells (Figure 4I), demonstrating higher \( \beta \)-AR sensitivity in cells isolated from unstressed GRK2KO mice.

To further clarify the mechanism responsible for the hypophosphorylation of PLB, we examined whether phosphodiesterase 4 (PDE4), which is activated by PKA, could...
play a mechanistic role. A PDE4-specific inhibitor, rolipram (10 mg/kg BW, i.p.), was injected into unstressed WT and GRK2KO mice for 2 hours to allow it to take effect. Then the hearts were snap frozen in liquid nitrogen, and Western blotting for phospho-PLB and total PLB was performed. Interestingly, rolipram blunted the hypophosphorylation of PLB in GRK2KO myocytes (Figure 4K and 4L), which indicates that the proposed higher PKA activity in GRK2KO myocytes results in an increased local PDE4 activity causing hypophosphorylation of PLB.

Loss of Myocyte GRK2 Before MI Prevents the Development of Heart Failure and Preserves Contractility of Myocytes

Our previous studies have shown that cardiac specific loss of GRK2 ameliorates the development of HF after MI.10 In vivo cardiac function as assessed by echocardiography 28 days after MI showed that although sham GRK2KO mice were indistinguishable from sham WT mice, GRK2KO mice had significantly improved post-MI cardiac function and ventricular remodeling after the loss of myocyte GRK2 (online-only Data Supplement Table I). Because this improved post-MI cardiac function was seen in GRK2KO mice with similar infarct sizes, the beneficial effects of GRK2 deficiency probably occurs at the myocyte level. Therefore, the function of cardiac myocytes isolated from WT and GRK2KO mice at 28 days after MI was determined. The basal fractional shortening and Ca\(^{2+}\) transient amplitudes in myocytes from GRK2KO mice after MI (GRK2KO MI) were almost normal compared with GRK2KO myocytes from mice not subject to MI and greater than those of WT cardiac myocytes after MI (WT MI) at both pacing frequencies of 0.5Hz and 2Hz (Figure 5A, B, D and E and online-only Data Supplement Table II). Furthermore, WT MI myocytes had a blunted functional response to ISO. In contrast, cardiac myocytes from GRK2KO mice after MI displayed significantly improved \(\beta\)-adrenergic responses (Figure 5A, B, D, and E). These results clearly show that the loss of GRK2 in cardiac myocytes can partially prevent pathological cellular mechanical and Ca\(^{2+}\)-handling remodeling after MI and provide a potential cellular mechanism for the benefits of GRK2 lowering or inhibition in the failing heart.

Figure 4. Silencing of GRK2 in cardiac myocytes increased NCX expression, reduced PLB phosphorylation by PKA-dependent PDE4 activation, and enhanced responsiveness to ISO. A, Tau values of caffeine-induced intracellular Ca\(^{2+}\) transient decay, indicating increased NCX activity in GRK2KO myocytes; n=16 to 29 cardiac myocytes from 3 different hearts. B, NCX currents at baseline in control (WT) and GRK2KO myocytes; n=4 animals/group, 2-way repeated ANOVA with posthoc Student t test. C, Western blot of NCX proteins in WT and GRK2KO hearts. D, NCX expression normalized to GAPDH. E and F, SERCA expression in WT and GRK2KO hearts. G and H, PLB expression and phosphorylation at Ser16 (PKA site) and Thr17 (CaMK II site) in WT and GRK2KO hearts; n=5 animals/group D, F, and H, t test for D, F, and H. I and J, PLB phosphorylation at Ser16 in response to different concentrations of ISO; n=5 animals/group; data were analyzed by regression with repeated measures for J. K and L, Western blotting for pSer16-PLB and total PLB in WT and GRK2KO hearts stimulated with or without the selective PDE4 inhibitor rolipram; a total of 3 to 5 hearts were analyzed for each group; 1-way ANOVA for L. WT indicates wild type; KO, knockout; ISO, isoproterenol; lNCX, Na\(^+\)-Ca\(^{2+}\) exchange current; NCX1, Na\(^+\)-Ca\(^{2+}\) exchange 1; SERCA, sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase; PLB, phospholamban; PLBt, total Phospholamban; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and N.S., not significant.
GRK2KO MI Myocytes Have Preserved SR Ca\textsuperscript{2+} Loading and I_{Ca,L} 

A decrease in the SR Ca\textsuperscript{2+} load is a contributing factor for depressed myocyte contractility, a hallmark of HF. Although GRK2KO mice without MI having a lower SR Ca\textsuperscript{2+} load than WT mice without MI, the SR Ca\textsuperscript{2+} load in GRK2KO MI myocytes was not decreased as in WT MI myocytes (Figure 6A and B and online-only Data Supplement Table II). Fractional Ca\textsuperscript{2+} release from the SR was higher in GRK2KO MI myocytes as well, explaining the preserved myocyte contractility (Figure 6C). Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger activity, as indirectly assessed by the decay constant tau of the caffeine-induced Ca\textsuperscript{2+} transient was normalized in post-MI GRK2KO myocytes, despite significant increases in NCX activity in infarcted WT mice consistent with severe HF (Figure 6D).

Improvements in intracellular Ca\textsuperscript{2+} transients and SR fractional Ca\textsuperscript{2+} release in the GRK2KO MI myocytes could result from changes in cardiac myocyte I_{Ca,L}. Although basal I_{Ca,L} amplitudes in both post-MI WT and GRK2KO myocytes were reduced compared with pre-MI values (see Figure 3A and 3B), peak I_{Ca,L} in post-MI myocytes were significantly greater with the loss of GRK2 (Figure 6E and online-only Data Supplement Table II). When stimulated with ISO (10^{-6} M), I_{Ca,L} in GRK2KO post-MI myocytes was only insignificantly increased (12.9±6.9%, n=4), but I_{Ca,L} in post-MI WT myocytes was significantly increased by 84.3±18.1% (n=7) (Figure 6F). However, after ISO, I_{Ca,L} amplitudes in myocytes were not different between the 2 mouse lines (Figure 6G and 6H), indicating similar LTCC density. The marked enhancement in basal I_{Ca,L} in the GRK2KO MI myocytes might contribute to the normalization of intracellular Ca\textsuperscript{2+} handling and improved cardiac myocyte contractility. Of interest, the ISO stimulation caused a significant leftward shift of voltage dependency of channel activation in WT post-MI myocytes but not in GRK2KO myocytes, probably because the activation of I_{Ca,L} in GRK2KO MI myocytes at baseline was already shifted to the left. After ISO stimulation, the voltage-dependent activation of I_{Ca,L} was similar in both groups (Figure 6G and 6H).

Loss of Myocyte GRK2 Inhibits Adverse Cellular Remodeling Post-MI

Cardiac myocyte size at 28 days post-MI was assessed by measurements of myocyte capacitance. Myocytes isolated from GRK2KO MI mice had significantly smaller capacitance, indicating less myocyte hypertrophy and inhibition of adverse cellular remodeling compared with WT mice post-MI (GRK2KO MI 211±19pF versus WT MI 311±35pF versus WT Sham 178±19pF versus KO Sham 188±15pF; P<0.05 between GRK2KO MI and WT MI).
Beneficial Effects of Myocyte GRK2 Silencing Is Suppressed by the LTCC Blocker Verapamil

To determine if the beneficial Ca$^{2+}$ effects seen post-MI after myocyte GRK2 lowering is mediated through the novel changes in LTCC function, we treated WT and GRK2KO mice with verapamil from 14 days till 42 days post-MI. Interestingly, echocardiography revealed that verapamil treatment negated some of the beneficial effects of GRK2 silencing on post-MI cardiac function whereas the LTCC blocker had minimal effects on post-MI WT mice (Figure 7A). Furthermore, cardiac brain natriuretic peptide messenger RNA expression as a molecular marker of HF was significantly lower in post-MI GRK2KO mice compared with WT MI mice but was reversed by verapamil treatment to the level seen in post-MI WT mice (Figure 7C).

Discussion

G-protein–coupled receptor kinase 2 is an important molecule in the heart. It is not only a primary regulator of adrenergic signaling but also claims an important role in the development of HF.8–10 It is upregulated during the early stage in injured myocardium, indicating that it participates in the progression of ventricular dysfunction and cardiomyopathy.8–10 Our previous studies have shown that GRK2 silencing10 or inhibition by βARKct20 is able to improve cardiac function during HF progression after MI. However, the specific role of GRK2 in the regulation of normal and failing Ca$^{2+}$ cycling has never been studied. In HF, myocyte Ca$^{2+}$ cycling is deranged, and abnormalities include altered cardiac LTCC density and properties and reduced SR Ca$^{2+}$ content due to decreased SERCA and increased NCX activities. These changes result in reduced intracellular Ca$^{2+}$ transients and depressed myocyte contractility.1,4 Our current study has revealed that GRK2 can influence myocyte Ca$^{2+}$ homeostasis and that its absence in cardiac myocytes10,13 causes a novel Ca$^{2+}$ handling phenotype that is resistant to cardiac function deterioration after MI. The benefits rendered by GRK2 silencing are associated with the differential regulation of sarcolemmal and SR Ca$^{2+}$ channel currents by β-adrenergic system.

A Novel Ca$^{2+}$-Handling Phenotype Induced by GRK2 Silencing

Although GRK2 plays an important role in regulating the β-adrenergic system, its loss does not affect basal cardiac10 and cardiomyocyte function. Myocyte loss of GRK2 did not change characteristics of basal intracellular Ca$^{2+}$ transients and myocyte contractions. However, detailed characterization of myocyte Ca$^{2+}$ handling has shown many differences in EC coupling between GRK2KO and WT myocytes: (1) The SR Ca$^{2+}$ content is reduced in GRK2 KO myocytes, but Ca$^{2+}$...
transients and contraction in GRK2KO myocytes are normal because of an increased fractional Ca\(^{2+}\) release from the SR; (2) increased IC\(_{Ca,L}\) ensures normal Ca\(^{2+}\) transients and cardiac myocyte contractility; (3) decreased SR Ca\(^{2+}\) content in the face of increased IC\(_{Ca,L}\) is due to increased Ca\(^{2+}\) efflux through the NCX and the inhibition of SERCA by hypophosphorylated PLB; and (4) increased IC\(_{Ca,L}\) is possibly due to local increase in PKA activity. Most of these aspects of Ca\(^{2+}\) handling in GRK2KO myocytes, except the greater than normal IC\(_{Ca,L}\) and enhanced \(\beta\)-adrenergic regulation, have some similarity with those observed in failing myocytes.\(^{21}\) These findings could imply that, even in failing myocytes, some of the Ca\(^{2+}\) handling aspects could be more of adaptive mechanisms.

**Differential Regulation of Sarcolemmal and SR Ca\(^{2+}\) Handling by the \(\beta\)-Adrenergic System**

Our data clearly show that there is a differential regulation of the LTCC on the sarcolemma and the PLB on the SR by the \(\beta\)-adrenergic system in GRK2KO myocytes: At baseline, the LTCC is already in high-activity mode probably because of the high-phosphorylation state of the channel, but the LTCC loses its responses to \(\beta\)-adrenergic stimulation; in contrast, the PLB is in a low-phosphorylation state (hypophosphorylation), but it has enhanced responsiveness to \(\beta\)-adrenergic receptor (\(\beta\)-AR) stimulation. Our study indicates that the increased LTCC activity could be due to an increase in subsarcolemmal (local) PKA activation brought about by constitutive activity of the \(\beta\)-ARs. In normal cardiac physiology, GRK2 mediates the desensitization of \(\beta\)-ARs.\(^{20}\) The loss of GRK2 prevents desensitization of \(\beta\)-ARs and thus likely promotes the accumulation of activated \(\beta\)-ARs and causes constitutive activity of \(\beta\)-ARs in GRK2KO myocytes even after the isolation. The increase in LTCC activity induced by constitutive \(\beta_1\)-ARs has been shown in cardiac \(\beta_1\)-AR–overexpression mice.\(^{18}\) The high LTCC activity in GRK2KO myocytes blunts the responsiveness of the channel to \(\beta\)-adrenergic stimulation. Similar situations have been reported in myocytes with high basal LTCC activities.\(^{17,22}\) Recently, we have shown that overexpression of \(\beta\)ARKct, an inhibitor of GRK2, in adult rat myocytes increases basal IC\(_{Ca,L}\) as we have seen with GRK2 silencing. However, \(\beta\)ARKct overexpression also enhanced the responses of IC\(_{Ca,L}\) to ISO,\(^{11}\) which is in contrast to our findings with GRK2KO. These results suggest that potentially different mechanisms are involved in our current study and the \(\beta\)ARKct study,\(^{11}\) with the net effect (increased LTCC) being comparable. Primarily, \(\beta\)ARKct reduces the inhibitory effect of the \(\beta_\gamma\) subunits of activated heterotrimeric-G proteins (G\(\beta\gamma\)) on the LTCC whereas GRK2KO leads to a local increase in PKA, thereby activating the LTCC. The role of G\(\beta\gamma\) in this setting was not specifically addressed, however: If G\(\beta\gamma\) was released with the KO of GRK2, the inhibitory effect on the LTCC must be at least overcome by the PKA-dependent activation of the LTCC. An interesting experiment for future studies will indeed be the expression of \(\beta\)ARKct in GRK2KO myocytes.
This experiment will finally address the role of $G_{\beta y}$ in this setting, but such a goal goes far beyond the scope of our current study. The use of different models (GRK2KO in mice in vivo for a relatively long period of time versus $\beta$ARKct expression in cultured rat ventricular myocytes\textsuperscript{11} in vitro for 24 hours) could also account for different mechanisms in mediating increased basal $I_{\text{Ca,L}}$ and different degrees of responsiveness to ISO stimulation. In contrast to the enhanced LTCC phosphorylation in GRK2KO myocytes, the phosphorylation state of PLB on the SR is lower than normal and the responsiveness of PLB to $\beta$-adrenergic agonists is enhanced. The underlying mechanism is related to locally activated PDE4 by activated subsarcolemmal PKA because rolipram, a selective PDE4 inhibitor, blunted the hypophosphorylation of PLB in GRK2KO hearts. Subsarcolemmal PDEs are generally able to diffuse to the SR and thus can limit local cAMP production and PKA activation.\textsuperscript{23} Our results mechanistically explain the differential regulation of sarcolemmal versus SR Ca\textsuperscript{2+} handling associated with the cardiac myocyte lowering of GRK2, which ultimately can improve cardiac function in HF models.

The Novel Ca\textsuperscript{2+}.-Handling Phenotype Induced by the Loss of GRK2 Is Resistant to Adverse Remodeling in Hearts After MI

As summarized above, GRK2KO induces a novel Ca\textsuperscript{2+}.-handling phenotype that maintains a normal myocyte contractility in a way that is less dependent on SR Ca\textsuperscript{2+} but more dependent on $I_{\text{Ca,L}}$. We also show that this type of Ca\textsuperscript{2+} handling in GRK2KO myocytes is more resistant to adverse remodeling induced by MI in that the SR Ca\textsuperscript{2+} content and its regulation by the $\beta$AR system and $I_{\text{Ca,L}}$ density are better preserved and NCX activity is not increased. The cellular processes responsible for better remodeling after MI in GRK2KO mice are potentially due to a combination of increased $I_{\text{Ca,L}}$ and normalized NCX activity resulting in reduced SR Ca\textsuperscript{2+} content at baseline. We suspect that the relatively unchanged and small NCX activity after MI predisposes the GRK2KO MI myocyte to maintain an unchanged SR Ca\textsuperscript{2+} loading, which is in contrast to WT mice. Overall, this combination renders cardiac myocytes less susceptible to SR Ca\textsuperscript{2+} overload, which is known to induce myocyte apoptosis and necrosis.\textsuperscript{24,25} Importantly, the beneficial effects of GRK2KO are negated by an LTCC antagonist, verapamil. Elevated SR Ca\textsuperscript{2+} content might also participate in myocyte hypertrophy.\textsuperscript{26} In this study, GRK2KO myocytes develop less cardiac myocyte hypertrophy post-MI. This could be due to the concomitant decrease of SR Ca\textsuperscript{2+} load induced by the loss of GRK2 expression in the cardiac myocyte. The enhanced $\beta$-adrenergic responsiveness in GRK2KO myocytes may also contribute to the beneficial effects of GRK2 silencing.

The results obtained here explain our previous studies showing that the loss of GRK2 in cardiac myocytes reduces HF-associated mortality and enhances global cardiac function post-MI.\textsuperscript{10} The improvements associated with the loss of GRK2 expression and activity are in large part attributable to the normalization of intracellular Ca\textsuperscript{2+} cycling and cardiac myocyte function.

Conclusions

In summary, our data provide novel and important insights into the role of GRK2 in normal and failing hearts. Loss of GRK2 in cardiac myocytes enhances ECC efficiency in the presence of a lower than normal SR Ca\textsuperscript{2+} loading condition and better $\beta$-adrenergic responsiveness in unstressed hearts. This enhancement of ECC occurs through differential regulation of sarcolemmal versus SR Ca\textsuperscript{2+} handling, with the net result being improved Ca\textsuperscript{2+} transients leading to the amelioration of the HF phenotype. This is seen at the myocyte level and also globally in vivo with improved cardiac function of GRK2KO mice post-MI. Our data revealed for the first time that the beneficial effects seen with a loss of myocyte GRK2 activity after MI were associated with marked amelioration of cardiac myocyte contractility and significant improvements in intracellular Ca\textsuperscript{2+} cycling effected by modulation of $I_{\text{Ca,L}}$. These effects seen at the myocyte level may also contribute to the beneficial effects seen in various HF models treated with the $\beta$ARKct peptide as a GRK2 inhibitor.\textsuperscript{8,9,12} Further, GRK2 appears to induce novel regulatory modulation in the LTCC because currents were enhanced with a loss of GRK2. Overall, our current results mechanistically explain the beneficial effects of GRK2 silencing or inhibition after MI in the heart at the cellular and molecular levels and validates GRK2 as a potential target for HF prevention and treatment.

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Disclosures

None.

References

1. Piacentino V III, Weber CR, Chen X, Weisser-Thomas J, Margulies KB, Bers DM, Houser SR. Cellular basis of abnormal calcium transients of failing human ventricular myocytes. Circ Res. 2003;92:651–658.
2. Houser SR, Piacentino V III, Weisser J. Abnormalities of calcium cycling in the hypertrophied and failing heart. J Mol Cell Cardiol. 2000;32:1595–1607.
3. Bers DM. Altered cardiac myocyte Ca regulation in heart failure. Physiol (Bethesda). 2006;21:380–387.
4. Chen X, Piacentino V III, Furukawa S, Goldman B, Margulies KB, Houser SR. L-type Ca\textsuperscript{2+} channel density and regulation are altered in failing human ventricular myocytes and recover after support with mechanical assist devices. Circ Res. 2002;91:517–524.
5. Bers DM. Calcium cycling and signaling in cardiac myocytes. Annu Rev Physiol. 2008;70:23–49.
6. Cohn JN, Levine TB, Oliviari MT, Garberg V, lura D, Francis GS, Simon AB, Rector T. Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. N Engl J Med. 1984;311:819–823.
7. Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane–spanning receptors and heart function. Nature. 2002;415:206–212.
8. Harding VB, Jones LR, Lefkowitz RJ, Koch WJ, Rockman HA. Cardiac beta ARK1 inhibition prolongs survival and augments beta blocker therapy in a mouse model of severe heart failure. Proc Natl Acad Sci U S A. 2001;98:5809–5814.
improved Ca\textsuperscript{2+} handling, with the net result being
unstressed hearts and in failing myocytes. We show that this enhancement of excitation-contraction coupling occurs through differential regulation of sarcoplasmic reticulum Ca\textsuperscript{2+} overload leading to reversal of the HF phenotype. Further, we demonstrate that the beneficial effects seen with GRK2 inhibition in HF were associated with marked amelioration of cardiac myocyte contractility and significant improvements in intracellular Ca\textsuperscript{2+} cycling effected by modulation of the cardiac L-type Ca\textsuperscript{2+} channels. A clearly novel finding is the compartmentalization of intracellular signaling by GRK2 and neurohormonal axis in chronic heart failure. Overall, our current results explain the beneficial effects of GRK2 inhibition in HF. We now provide the molecular basis for potential benefits of therapeutic strategies aiming at GRK2 inhibition. With development and beta-adrenergic signaling. Circulation. 2006;99:996–1003.

10. Most P, Seifert H, Gao E, Funakoshi H, Volkers M, Heierhorst J, Schroder F, Handrock R, Beuckelmann DJ, Hirt S, Hullin R, Priebe L. Inotropic and mitochondrial-dependent cardiomyocyte necrosis as a primary mediator of heart failure. J Clin Invest. 2007;117:2431–2444.

11. Tang M, Zhang X, Li Y, Guan Y, Ai X, Szeto C, Nakayama H, Zhang H, Ge S, Molkentin JD, Houser SR, Chen X. Enhanced basal contractility but reduced excitation–contraction coupling efficiency and beta-adrenergic reserve of hearts with increased cav1.2 activity. Am J Physiol Heart Circ Physiol. 299:H519–H528.

12. Rengo G, Lympertzopoulos A, Zincarelli C, Donniaco M, Soltys S, Rabinowitz JE, Koch WJ. Myocardial adeno-associated virus serotype 6-betaARKct gene therapy improves cardiac function and normalizes the neurohormonal axis in chronic heart failure. Circulation. 2009;119:89–98.

13. Rockman HA, Chien KR, Choi DJ, Iaccarino G, Hunter JJ, Ross J Jr, Heierhorst J, Baines CP, Klevitsky R, Zhang X, Zhang H, Jaleel KP. Prevention of cardiomyopathy in mouse models lacking the smooth muscle sarcoglycan-sarcospan complex. J Clin Invest. 2001;107:R1–R7.

14. Cohn RD, Durbeej M, Moore SA, Coral-Vazquez R, Proulx S, Campbell KP. Prevention of cardiomyopathy in mouse models lacking the smooth muscle sarcoglycan-sarcospan complex. J Clin Invest. 2001;107:R1–R7.

15. Bers DM. Cardiac excitation–contraction coupling. Nature. 2002;415:198–205.

16. Schroder F, Handrock R, Beuckelmann DJ, Hirt S, Hullin R, Prieb L, Schwinger RH, Weil J, Herzog S. Increased availability and open probability of single L-type calcium channels from failing compared with nonfailing human ventricle. Circulation. 1998;98:969–976.

17. Foerster K, Kaeferstein T, Groner F, Engelhardt S, Mathies J, Koch WJ, Rohde MJ, Herzog S. Cardiac channel function and regulation in beta 1– and beta 2-adrenoceptor transgenic mice. Naunyn Schmiedebergs Arch Pharmacol. 2004;369:490–495.

18. Tang M, Zhang X, Li Y, Guan Y, Ai X, Szeto C, Nakayama H, Zhang H, Ge S, Molkentin JD, Houser SR, Chen X. Enhanced basal contractility but reduced excitation–contraction coupling efficiency and beta-adrenergic reserve of hearts with increased cav1.2 activity. Am J Physiol Heart Circ Physiol. 299:H519–H528.

19. De Arcangelis V, Liu S, Zhang D, Soto D, Xiang YK. Equilibrium between adenyl cyclase and phosphodiesterase patterns adrenergic agonist dose-dependent spatiotemporal cAMP/ protein kinase A activities in cardiomyocytes. Mol Pharmacol. 2010;78:340–349.

20. Muth JN, Bodi I, Lewis W, Varadi G, Schwartz A. A Ca(2+)–dependent transgenic model of cardiac hypertrophy: a role for protein kinase Calpha. Circulation. 2001;103:140–147.

21. De Arcangelis V, Liu S, Zhang D, Soto D, Xiang YK. Equilibrium between adenyl cyclase and phosphodiesterase patterns adrenergic agonist dose-dependent spatiotemporal cAMP/protein kinase A activities in cardiomyocytes. Mol Pharmacol. 2010;78:340–349.

22. Schroder F, Handrock R, Beuckelmann DJ, Hirt S, Hullin R, Prieb L, Schwinger RH, Weil J, Herzog S. Increased availability and open probability of single L-type calcium channels from failing compared with nonfailing human ventricle. Circulation. 1998;98:969–976.

23. De Arcangelis V, Liu S, Zhang D, Soto D, Xiang YK. Equilibrium between adenyl cyclase and phosphodiesterase patterns adrenergic agonist dose-dependent spatiotemporal cAMP/protein kinase A activities in cardiomyocytes. Mol Pharmacol. 2010;78:340–349.

24. Muth JN, Bodi I, Lewis W, Varadi G, Schwartz A. A Ca(2+)–dependent transgenic model of cardiac hypertrophy: a role for protein kinase Calpha. Circulation. 2001;103:140–147.

25. Nakayama H, Chen X, Banas CP, Klevitsky R, Zhang X, Zhang H, Jaleel N, Chua BH, Hewett TE, Robbins J, Houser SR, Molkentin JD. Ca(2+–)- and mitochondrial-dependent cardiomyocyte necrosis as a primary mediator of heart failure. J Clin Invest. 2007;117:2431–2444.

26. Chen X, Nakayama H, Zhang X, Ai X, Harris DM, Tang M, Zhang H, Szeto C, Stockbower K, Berretta RM, Eckhart AD, Koch WJ, Molkentin JD, Houser SR. Calcium influx through Cav1.2 is a proximal signal for pathological cardiomyocyte hypertrophy. J Mol Cell Cardiol. 2011;50:460–470.