Cardiac G-protein-coupled receptor kinase 2 ablation induces a novel Ca2+ handling phenotype resistant to adverse alterations and remodeling after myocardial infarction.

Philip W Raake  
Department of Internal Medicine III, Cardiology, University of Heidelberg

Xiaoying Zhang  
Cardiovascular Research Center, Department of Physiology, Temple University School of Medicine

Leif E Vinge  
Center for Translational Medicine, Department of Medicine, Thomas Jefferson University

Henriette Brinks  
Center for Translational Medicine, Department of Medicine, Thomas Jefferson University

Erhe Gao  
Center for Translational Medicine, Department of Pharmacology, Temple University

See next page for additional authors

Recommended Citation  
Raake, Philip W; Zhang, Xiaoying; Vinge, Leif E; Brinks, Henriette; Gao, Erhe; Jaleel, Naser; Li, Yingxin; Tang, Mingxin; Most, Patrick; Dorn, Gerald W; Houser, Steven R; Katus, Hugo A; Chen, Xiongwen; and Koch, Walter J, "Cardiac G-protein-coupled receptor kinase 2 ablation induces a novel Ca2+ handling phenotype resistant to adverse alterations and remodeling after myocardial infarction." (2012). Center for Translational Medicine Faculty Papers. Paper 17.  
https://jdc.jefferson.edu/transmedfp/17

This Article is brought to you for free and open access by the Jefferson Digital Commons. The Jefferson Digital Commons is a service of Thomas Jefferson University’s Center for Teaching and Learning (CTL). The Commons is a showcase for Jefferson books and journals, peer-reviewed scholarly publications, unique historical collections from the University archives, and teaching tools. The Jefferson Digital Commons allows researchers and interested readers anywhere in the world to learn about and keep up to date with Jefferson scholarship. This article has been accepted for inclusion in Center for Translational Medicine Faculty Papers by an authorized administrator of the Jefferson Digital Commons. For more information, please contact: JeffersonDigitalCommons@jefferson.edu.
Cardiac G-Protein–Coupled Receptor Kinase 2 Ablation Induces a Novel Ca^{2+} Handling Phenotype Resistant to Adverse Alterations and Remodeling After Myocardial Infarction

Philip W. Raake, Xiaoying Zhang, Leif E. Vinge, Henriette Brinks, Erhe Gao, Naser Jaleel, Yingxin Li, Mingxin Tang, Patrick Most, Gerald W. Dorn II, Steven R. Houser, Hugo A. Katus, Xiongwen Chen and Walter J. Koch

_Circulation_. 2012;125:2108-2118; originally published online April 10, 2012;
doi: 10.1161/CIRCULATIONAHA.111.044255

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/125/17/2108

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2012/04/06/CIRCULATIONAHA.111.044255.DC1.html

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/
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

Philip W. Raake, MD; Xiaoying Zhang, PhD; Leif E. Vinge, MD, PhD; Henriette Brinks, MD; Erhe Gao, MD, PhD; Naser Jaleel, MS; Yingxin Li, MS; Mingxin Tang, MS; Patrick Most, MD; Gerald W. Dorn II, MD; Steven R. Houser, PhD; Hugo A. Katus, MD; Xiongwen Chen, PhD; Walter J. Koch, PhD

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 cardiomyocytes isolated from unstressed 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+} exchange 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

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

A

heart failure

Circulation is available at http://circ.ahajournals.org

Received June 4, 2011; accepted March 2, 2012.
From the Department of Internal Medicine III, Cardiology, University of Heidelberg, Heidelberg, Germany (P.W.R., H.A.K.); Cardiovascular Research Center/Department of Physiology (X.Z., N.J., Y.L., M.T., S.R.H., X.C.), and Center for Translational Medicine, Department of Pharmacology (E.G., W.J.K.), Temple University; Center for Translational Medicine, Department of Medicine, Thomas Jefferson University (L.E.V., H.B., P.M.), Philadelphia, PA; and Center for Pharmacogenomics, Washington University, St. Louis, MO (G.W.D.).

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.111.044255/-/DC1.

Drs Raake and Zhang contributed equally to this article.
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 β-adrenergic signaling pathway. In acute HF, these changes can improve systemic perfusion whereas in chronic HF the augmentation in catecholamines is associated with mortality and results in a downregulation of β-adrenergic receptors (βARs) 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 βAR signaling. We have previously identified GRK2 as a culprit in the progression of HF, and GRK2 inhibition (by expression of its c-terminal domain, called βARKct) or gene silencing has rescued disparate models of HF. The recent study indicates that the benefits of βARKct could be related to enhanced myocyte contractility by increasing LTCC currents and its responsiveness to β-adrenergic agonists. However, the exact underlying cellular mechanisms for these beneficial effects in HF after βARKct expression or GRK2 ablation are not clearly defined. It is especially important to define these mechanisms because in light of the recent success of βAR blocker therapy in clinical HF management, the results with βARKct and GRK2 silencing appear paradoxical, as the major function of GRK2 in cardiac myocytes is to dampen βAR signaling in a manner similar to that of βAR blockade. We have found that βARKct expression can cause a molecular remodeling of the cardiac βAR system with receptor upregulation and improved βAR signaling, and a recent study with chronic mediated βARKct expression in a rat HF model showed that myocardial βAR changes are probably down-stream of neurohormonal lowering including reduction in sympathetic nervous system activity. In this regard, the role of GRK2 inhibition must mechanistically go beyond resensitizing βARs 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 cardiomyocyte-specific GRK2KO mice 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\(_{\text{Ca,L}}\)) 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. G-protein–coupled receptor kinase 2 KO (α myosin heavy chain – Cre-recombinase × GRK2flox/flox) and wild-type (WT) (GRK2flox/flox) mice were maintained on a C57BL6 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. 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 and were evaluated by echocardiography. Cardiac myocytes were isolated cultured from animals. Myocyte Ca\(^{2+}\) transients and contractions, SR Ca\(^{2+}\) load, I\(_{\text{Ca,L}}\) and NCX-Activity I\(_{\text{NCX}}\) 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±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 I), but their cardiac function responds better to β-adrenergic stimulation. 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 βAR 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 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-1AM) 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 $I_{\text{Ca,L}}$ 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 $I_{\text{Ca,L}}$ in GRK2KO and control myocytes because $I_{\text{Ca,L}}$ serves as both the trigger of Ca$^{2+}$ release from the SR and the source for loading the SR.16 Peak $I_{\text{Ca,L}}$ 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 $I_{\text{Ca,L}}$ was increased to the same level in cardiac myocytes from both mouse lines (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 $I_{\text{Ca,L}}$, properties, we tested whether the increased $I_{\text{Ca,L}}$ 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 density on the membrane and that the increased whole-cell $I_{\text{Ca,L}}$ 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 $I_{\text{Ca,L}}$ 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.18 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 $I_{\text{Ca,L}}$ 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 /H9252 AR 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 /H9252 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 4J), demonstrating higher /H9252 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\(^{2+}\)/H\(^{1+}\) 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.
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 have 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\(^{2+}\) 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). It could be true that the absolute amount of Ca\(^{2+}\) 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\(^10\) or inhibition by \(\beta\)ARKct\(^20\) 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 myocytes\(^10,13\) 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\(^{2+}\) handling by the \(\beta\)-adrenergic system.

A Novel Ca\(^{2+}\)-Handling Phenotype Induced by GRK2 Silencing

Although GRK2 plays an important role in regulating the \(\beta\)-adrenergic system, its loss does not affect basal cardiac\(^10\) 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 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 \(\beta\)-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 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 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 \(\beta_2\) 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 $G_{protein}$ 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 βARKct expression in cultured rat ventricular myocytes\textsuperscript{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 β-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 $I_{Ca,L}$. 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 βAR 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\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{24–28} 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 β-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 β-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 $I_{Ca,L}$. These effects seen at the myocyte level may also contribute to the beneficial effects seen in various HF models treated with the βARKct 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.

Sources of Funding

This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (Ra 1668/1-1 and Ra 1668/3-1 to Dr Raake). Drs Chen, Li, and Tang were supported by National Institutes of Health (NIH) grant R01 HL088243 and American Heart Association grant AHA07030437N. Dr Koch is the W.W. Smith Professor of Medicine, and this research was supported by NIH grants R37 HL61690, R01 HL56205, R01 HL085503, and P01 HL075443 (Project 2). Also, Drs. Koch and Gao were supported through P01 HL091799 (Project 1 and Core B). The effort of Dr Dorn II on this project was supported by NIH grant R01 HL87871, and Dr Houser was supported by NIH grant P01 HL091799 (Project 3).

Disclosures

None.

References


**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 unstrained 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 thereby differential regulation of Ca2+ signaling. 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.
SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Experimental animals
Conditional mice bearing floxed GRK2 alleles were described previously\(^1\), \(^2\). GRK2KO (\(\alpha\)MHC-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 \(\text{Ca}^{2+}\) 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 \(\alpha\)-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 \(\alpha\)-MEM with Hank’s salt solution supplemented with L-Glutamine (10 mmol/l), 2, 3-Butandionemonoxime (BDM, 10mmol/l)
and Insulin-Transferrin-Selenium (1:1000 dilution) (Gibco, Invitrogen Corporation, Carlsbad, California, USA) with a humidified atmosphere containing 2% CO$_2$ 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.

**Ca$^{2+}$ Transients and single myocyte contractility studies**

Freshly isolated cells were kept in BDM-free tyrodes solution containing 1mM CaCl$_2$. 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 CaCl$_2$ without (baseline) and with $10^{-8}$ M Isoproterenol (Sigma-Aldrich, St. Louis, Missouri, USA). Ca$^{2+}$ 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 Ca$^{2+}$ load**

Myocytes were placed in a chamber mounted on an inverted Nikon microscope and perfused with normal physiological solution (Tyrode) containing 1mM Ca$^{2+}$. Since MI may cause the increase of diastolic intracellular Ca$^{2+}$, Indo-1 AM was used to measure Ca$^{2+}$ transients and caffeine-induced Ca$^{2+}$ transients for myocytes isolated from sham and post-MI hearts as described previously $^6$-$^8$. To assess SR Ca$^{2+}$ 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 Ca$^{2+}$ transient was used as the index of SR content. Peak Ca$^{2+}$ concentrations of Ca$^{2+}$ transients induced by field stimulation and caffeine
spritz were calculated as follows: \[ [\text{Ca}^{2+}] = \frac{K_d \times R}{((K_d / \text{[Ca}^{2+}])_{\text{rest}} + 1) - R}, \]

where \( R \) is the ratio of emitted fluorescence to the resting emitting fluorescence. \([\text{Ca}^{2+}]_{\text{rest}}\) 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 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 Ca\( ^{2+} \) 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 (5\( \mu \)M) 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 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. 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, 5\( \mu \)M) on \( I_{\text{Ca,L}} \), 5\( \mu \)M 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 (+80 mV to -80 mV at 100 mV/s) following a 100-ms depolarization to +80 from the holding potential of -40 mV. During the recording, the bath solution then was changed to the K\textsuperscript{+}-free solution with 5 mmol/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 p1928α1c 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\(^2\) 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

<table>
<thead>
<tr>
<th></th>
<th>Sham WT</th>
<th>Sham GRK2KO</th>
<th>MI WT</th>
<th>MI GRK2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate [beats/min]</td>
<td>471±22</td>
<td>452±14</td>
<td>415±9</td>
<td>433±12</td>
</tr>
<tr>
<td>End-Diastolic Diameter [mm]</td>
<td>3,08±0,12</td>
<td>3,14±0,16</td>
<td>4,44±0,13</td>
<td>4,03±0,09 *</td>
</tr>
<tr>
<td>End-Systolic Diameter [mm]</td>
<td>1,81±0,09</td>
<td>1,87±0,12</td>
<td>3,63±0,13</td>
<td>3,03±0,09 *</td>
</tr>
<tr>
<td>Fractional Shortening [%]</td>
<td>41,41±1,53</td>
<td>40,60±1,03</td>
<td>18,21±0,94</td>
<td>24,88±1,34 *</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th></th>
<th>Sham WT</th>
<th>Sham GRK2KO</th>
<th>MI WT</th>
<th>MI GRK2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS at 0.5Hz [%]</td>
<td>6.24±0.42</td>
<td>7.16±0.44</td>
<td>2.56±0.19</td>
<td>4.19±0.14</td>
</tr>
<tr>
<td>[Ca²⁺], peak at 0.5Hz [Fura-2,340/380]</td>
<td>0.091±0.005</td>
<td>0.103±0.004</td>
<td>0.075±0.002</td>
<td>0.108±0.003</td>
</tr>
<tr>
<td>FS at 2Hz [%]</td>
<td>4.90±0.51</td>
<td>6.43±0.58</td>
<td>3.53±0.48</td>
<td>5.65±0.35</td>
</tr>
<tr>
<td>[Ca²⁺], peak at 2Hz [Fura-2, 340/380]</td>
<td>0.078±0.003</td>
<td>0.089±0.004</td>
<td>0.048±0.006</td>
<td>0.084±0.005</td>
</tr>
<tr>
<td>SR Ca²⁺ load [indo-1, 410/480]</td>
<td>0.26±0.02</td>
<td>0.22±0.01</td>
<td>0.15±0.01</td>
<td>0.26±0.04</td>
</tr>
<tr>
<td>Fractional Ca²⁺ release [%]</td>
<td>64.4±1.3</td>
<td>75.4±4.4</td>
<td>70.6±6.1</td>
<td>83.9±5.9</td>
</tr>
<tr>
<td>t_{Ca,L} peak [pA/pF]</td>
<td>-10.8±2.0</td>
<td>-18.57±0.9</td>
<td>-3.5±1.3</td>
<td>-5.0±1.5</td>
</tr>
</tbody>
</table>

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⁺]i peak at 0.5Hz [Fura-2,340/380]) and 2.0 Hz ([Ca²⁺]i 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 I_{Ca,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


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 sarcolemmal Na+Ca2+ exchanger in failing pig myocytes. Circulation research. 2003;92:897-903

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


10. Chen X, Piacentino 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