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Cardiac GRK2 Ablation Induces a Novel Ca²⁺ Handling Phenotype Resistant to Adverse Alterations and Remodeling After Myocardial Infarction

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Abstract

Background—G-protein coupled receptor kinase 2 (GRK2) is a primary regulator of β -adrenergic signaling in the heart. GRK2 ablation ameliorates heart failure development but the cellular mechanisms have not been elucidated and are the aim of this study.

Methods and Results—Myocyte contractility, Ca²⁺ handling and excitation-contraction coupling were studied in isolated cardiomyocytes from wild-type (WT) and GRK2 knockout (GRK2KO) mice without (sham) or with myocardial infarction (MI). In cardiac myocytes isolated from unstressed WT and GRK2KO hearts, myocyte contractions and Ca²⁺ transients were similar but GRK2KO myocytes had lower sarcoplasmic reticulum (SR) Ca²⁺ content due to increased sodium-Ca²⁺ exchanger (NCX) activity and inhibited SR Ca²⁺ ATPase by local PKA mediated activation of PDE4 resulting in hypophosphorylated phospholamban. This Ca²⁺ handling phenotype is explained by a higher fractional SR Ca²⁺ release induced by increased L-type Ca²⁺ channel currents (I_{Ca,L}). After β -adrenergic stimulation, GRK2KO myocytes revealed significant increases in contractility and Ca²⁺ transients, which were not mediated through cardiac L-type Ca²⁺ channels (LTCCs), but through an increased SR Ca²⁺. Interestingly, post-MI GRK2KO mice showed better cardiac function than post-MI control mice, which is explained by an improved Ca²⁺ handling phenotype. The SR Ca²⁺ content was better maintained in post-MI GRK2KO myocytes than in post-MI control myocytes, due to better maintained I_{Ca,L} density and no increase in NCX in GRK2KO myocytes. An LTCC blocker, verapamil, reversed some beneficial effects of GRK2KO.

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Conclusions—These data argue for novel differential regulation of I_{CaL} and SR load by GRK2. GRK2 ablation represents a novel beneficial Ca^{2+} handling phenotype resisting adverse remodeling after MI.

Keywords

calcium; experimental models heart failure; heart failure; Excitation Contraction Coupling; G-Protein Coupled Receptor Kinase 2

Introduction

Alterations in myocyte contractility and Ca^{2+} cycling are hallmarks of the progression of heart failure (HF). In failing cardiac myocytes, the SR Ca^{2+} content is decreased and the Ca^{2+} transient is decreased and prolonged, resulting in depressed myocyte contractility¹. This is generally considered to be a consequence of increased sodium– Ca^{2+} exchanger (NCX) activity, reduced sarcoplasmic (SR) reticulum Ca^{2+} ATPase (SERCA2a) expression and activity (probably due to decreased phospholamban (PLB) phosphorylation), and increased PLB/SERCA2a ratio, as well as an augmented open probability of the ryanodine receptor (RyR), 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 myocytes⁴. 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 remodeling⁵.

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 mortality⁶ and results in a down-regulation of β -adrenergic receptors (β ARs) promoted by upregulated G protein-coupled receptor kinase 2 (GRK2). GRK2 is the primary GRK in the heart and a prototype regulator of β AR signaling⁷. We have previously identified GRK2 as a culprit in the progression of HF and GRK2 inhibition (by expression of its c-terminal domain, called β ARKct) or gene silencing has rescued disparate models of HF⁸⁻¹⁰. Our recent study indicates the benefits of β ARKct could be related to enhanced myocyte contractility by increasing LTCC currents and its responsiveness to β -adrenergic agonists¹¹. However, the exact underlying cellular mechanisms for these beneficial effects in HF after β ARKct expression or GRK2 ablation are not clearly defined. It is especially important to define these mechanisms since in light of the recent success of β AR blocker therapy in clinical HF management, the results with β ARKct and GRK2 silencing appear paradox, as the major function of GRK2 in cardiac myocytes is to dampen β AR signaling, similar to β AR blockade. We have found that β ARKct expression can cause a molecular remodeling of the cardiac β AR system with receptor up-regulation and improved β AR signaling, and a recent study with chronic AAV-mediated β ARKct expression in a rat HF model showed that myocardial β AR changes are probably down-stream of neurohormonal lowering including reduction in sympathetic nervous system activity¹². In this regard, the role of GRK2 inhibition must mechanistically go beyond resensitizing β 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

GRK2 knockout mice^{10, 13} to study myocyte ECC coupling and Ca²⁺ 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 Ca²⁺ handling phenotype: myocyte contractility and Ca²⁺ handling are normal even though the SR Ca²⁺ content is reduced because there is an increased LTCC activities and resulting increases in I_{Ca,L} with a compensatory increase in NCX activity. This Ca²⁺ handling phenotype brought about by GRK2 ablation is resistant to adverse Ca²⁺ handling remodeling after MI and leads to better cardiac function in GRK2 KO mice post MI.

Materials and Methods

Conditional mice bearing floxed GRK2 alleles were described previously^{10, 13}. GRK2KO (α MHC-Cre \times 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. GRK2KO and WT mice were 8-10 weeks of age when entering the study. Unstressed normal mice and mice with coronary artery ligation (myocardial infarction (MI)) or sham-operation were studied. MI was induced by ligating the left anterior descending coronary artery at 2-3 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 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) as described previously¹⁵ and were evaluated by echocardiography (ECHO). Cardiac myocytes were isolated cultured from animals. Myocyte Ca²⁺ transients and contractions, SR Ca²⁺ load, LTCC currents (I_{Ca,L}), and I_{NCX} were measured. Quantitative real-time polymerase chain reaction and Western blot analysis were done for gene expression assessment. Detailed description of experimental procedures is available in the expanded **online-only data supplement**.

Data are expressed as mean \pm 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.

Results

Myocyte GRK2 ablation enhances adrenergic responsiveness of cellular contractility and Ca²⁺ transients

Our previous study has shown that GRK2 KO mice have comparable cardiac function as control mice at baseline (Supplemental Table 1) but their cardiac function responds to β -adrenergic stimulation better¹⁰. Here we determine the cellular mechanisms for this observation. Myocyte contraction and intracellular Ca²⁺ transients were recorded from WT and GRK2KO myocytes under baseline conditions and after β AR stimulation. Myocytes from both lines revealed a similar fractional shortening when paced at both 0.5Hz and 2Hz (Figure 1A-C). However, after isoproterenol (ISO), myocytes from GRK2 KO mice showed a significantly greater increase in fractional shortening (Figure 1A-C, Supplemental Table 2). In a good agreement, as shown in Figure 1D, at baseline, the characteristics and amplitude of the Ca²⁺ transients (as β Fura-2 340/380nm 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-F, Supplemental Table 2). We observed no differences in the Ca²⁺ transient decay time 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 SR¹⁶. We measured SR Ca^{2+} content by 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 a rapid application of caffeine (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 is 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 to WT myocytes at both baseline and after ISO (Figure 2C, Supplemental Table 2).

Loss of myocyte GRK2 enhances $I_{\text{Ca,L}}$ by a local PKA-dependent mechanism but blunts its β AR responsiveness

To explore the underlying cellular mechanisms for the reduced SR Ca^{2+} content while enhanced EC coupling efficiency in GRK2 KO myocytes, we measured the LTCC current ($I_{\text{Ca,L}}$) in GRK2 KO 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 to WT cardiac myocytes (Figure 3A and B, Supplemental Table 2). 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 3A and B), suggesting that the LTCC density was similar in both groups⁴. The voltage-dependency 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 D). 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 and/or the increase of channel activities at single channel levels. Charge movement was used to quantitate the amount 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 induced by various depolarizing voltages, indicating that there was no significant alteration of LTCC density on the membrane and the increased whole-cell $I_{\text{Ca,L}}$ could be due to the increased single channel activities. Immunoprecipitation of $\alpha 1c$, the pore forming subunit of the LTCC, from the same amount of proteins from GRK2KO and control hearts showed no difference in $\alpha 1c$ expression (Figure 3N). These data suggest that the LTCC in GRK2KO myocytes must have higher than normal activity, which was supported by single channel recording of LTCC activities (Figure 3J-M). The availability (Figure 3K) and the open probabilities (Figure 3L and M) were significantly increased in GRK2KO myocytes, which 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 β ARs carries constitutive activation to activate PKA locally to phosphorylate the LTCC¹⁸. Here we tested if 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-I). 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-M). The phosphorylation of α_1c at Ser1928, a PKA site, was shown to be more in GRK2KO hearts than in control hearts (Figure 3N and O).

Loss of GRK2 in myocytes increases $\text{Na}^+/\text{Ca}^{2+}$ exchanger expression

Since 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 sodium- Ca^{2+} exchanger (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 equation¹⁹. The tau value was significantly smaller in the GRK2 KO 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 to WT myocytes, which was confirmed by direct measurement of NCX current (Figure 4B) and NCX protein expression (Figure 4C and D).

Loss of myocytes GRK2 decreases basal phospholamban (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 PLB¹⁶. 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-H). 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 H). 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 J). However, a significant leftward shift of the dose response curve was found in GRK2KO myocytes compared to 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 PDE 4, which is activated by PKA, could play a mechanistic role. A PDE 4 specific inhibitor rolipram (10mg/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 (Figures 4K and L), 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 myocytes contractility

Our previous studies have shown that cardiac specific loss of GRK2 ameliorates the development of heart failure after MI¹⁰. *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 (Supplemental Table 1). Since 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 were determined. The basal fractional shortening (FS) and Ca^{2+} transient amplitudes in myocytes from GRK2KO mice post MI (GRK2KO MI) were almost

normal compared to GRK2KO myocytes from mice not subject to MI and greater than those of WT cardiac myocytes post-MI (WT MI) at both pacing frequencies of 0.5Hz and 2Hz (Figure 5A, B, D and E, Supplemental Table 2). Furthermore, WT MI myocytes had a blunted functional response to ISO. In contrast, cardiac myocytes from GRK2KO mice post-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 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 the 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, Supplemental Table 2). Fractional Ca^{2+} release from the SR was higher in GRK2KO MI myocytes as well, explaining the preserved myocyte contractility (see above and Figure 6C). NCX-activity, as indirectly assessed by the decay constant tau of the caffeine-induced peak Ca^{2+} transient was normalized in post-MI GRK2KO myocytes, despite significant increases in NCX activity in infarcted WT mice consistent with severe heart failure (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 to pre-MI values (see Figure 3A and B), peak $I_{\text{Ca,L}}$ in post-MI myocytes were significantly greater with the loss of GRK2 (Figure 6E, Supplemental Table 2). When stimulated with ISO (10^{-6}M), $I_{\text{Ca,L}}$ in GRK2KO post-MI myocytes was only insignificantly increased ($12.9\pm 6.9\%$, $n=4$) but $I_{\text{Ca,L}}$ in post-MI WT myocytes was significantly increased by $84.3\pm 18.1\%$ ($n=7$) (Figure 6F). However, after ISO, $I_{\text{Ca,L}}$ amplitudes in myocytes were not different between the two mouse lines (Figure 6G and H), 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 H).

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 to WT mice post-MI (GRK2KO MI $211\pm 19\text{pF}$ vs. WT MI $311\pm 35\text{pF}$ vs. WT Sham $178\pm 19\text{pF}$ vs. KO Sham $188\pm 15\text{pF}$; $p<0.05$ 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 while the LTCC blocker had minimal effects on post-MI WT mice (Figure 7A). Furthermore, cardiac BNP mRNA expression as a molecular marker of HF was significantly lower in post-MI GRK2KO mice compared to 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 $\text{I}_{\text{Ca,L}}$.

Discussion

GRK2 is an important molecule in the heart not only as a primary regulator of adrenergic signaling but also claims an important role in the development of HF⁸⁻¹⁰. It is up-regulated during the early stage in injured myocardium, indicating that it participates in the progression of ventricular dysfunction and cardiomyopathy⁸⁻¹⁰. Our previous studies have shown that GRK2 silencing¹⁰ or inhibition by βARKct^{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 heart failure, myocyte Ca^{2+} cycling is deranged, and abnormalities include altered cardiac LTCC density and properties and reduced SR Ca^{2+} content due to decreased SERCA2a 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 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 is associated with the differential regulation of sarcolemmal and SR Ca^{2+} handling by the β -adrenergic system.

1. A novel Ca^{2+} handling phenotype induced by GRK2 silencing

Although GRK2 plays an important role in regulating the β -adrenergic system, its KO 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 have shown many differences in ECC coupling between GRK2KO and WT myocytes: (1) The SR Ca^{2+} content is reduced in GRK2 KO myocytes but Ca^{2+} transient and contraction in GRK2KO myocytes are normal because an increased fractional Ca^{2+} release from the SR; (2) Increased $\text{I}_{\text{Ca,L}}$ ensures normal Ca^{2+} transients and cardiac myocyte contractility; (3) Decreased SR Ca^{2+} content in the face of increased $\text{I}_{\text{Ca,L}}$ is due to increased Ca^{2+} efflux through the NCX and the inhibition of SERCA by hypophosphorylated PLB; (4) Increased $\text{I}_{\text{Ca,L}}$ is possibly due to local increase in PKA activity. Most of these aspects of Ca^{2+} handling in GRK2 KO myocytes, except the greater than normal $\text{I}_{\text{Ca,L}}$ and enhanced β -adrenergic regulation, have some similarity to these observed in failing myocytes²¹. These findings could imply that even in failing myocytes, some of the Ca^{2+} handling aspects could be more of adaptation.

2. Differential regulation of sarcolemmal and SR Ca^{2+} handling by the β -adrenergic system

Our data clearly show that there is a differential regulation of the LTCC and the PLB on the SR by the β -adrenergic system in GRK2 KO myocytes: at baseline, the LTCC is already in high activity mode probably due to high phosphorylation state of the channel but the LTCC loses its responses to β -adrenergic stimulation; in contrast, the PLB is in low phosphorylation state (hypophosphorylation) but it has enhanced responsiveness to β -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 β -

ARs. In normal cardiac physiology, GRK2 mediates the desensitization of β -ARs²⁰. The loss of GRK2 prevents desensitization of β -ARs and thus likely promotes the accumulation of activated β -ARs and causes constitutive activity of β -ARs in GRK2 KO myocytes even after the isolation. The increase in LTCC activity induced by constitutive β_1 -ARs has been shown in cardiac β_1 -AR overexpression mice¹⁸. The high LTCC activity in GRK2KO myocytes blunts the responsiveness of the channel to β -adrenergic stimulation. Similar situations have been reported in myocytes with high basal LTCC activities^{17, 22}. Recently, we have shown that overexpression of β ARKct, an inhibitor of GRK2, in adult rat myocytes increases basal $I_{Ca,L}$ as we have seen with GRK2 silencing. However, β ARKct overexpression also enhanced the responses of $I_{Ca,L}$ to ISO¹¹, which contrasts to our findings with GRK2 KO. These results suggest a potentially different mechanism is involved in our current study and the β ARKct study¹¹ with the net effect (increased LTCC) being comparable. β ARKct mainly reduces the inhibitory $G_{\beta\gamma}$ effect on the LTCC while in contrast, 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 β ARKct in GRK2KO myocytes. This experiment will finally address the role of $G_{\beta\gamma}$ in this setting but 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 vs. β ARKct expression in cultured rat ventricular myocytes¹¹ in vitro for 24 hours) could also account for different mechanisms in mediating increased basal $I_{Ca,L}$ and different responsiveness to ISO stimulation.

In contrast to the enhanced LTCC phosphorylation in GRK2 KO 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 since 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. 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.

3. The novel Ca^{2+} handling phenotype induced by the loss of GRK2 resists to adverse remodeling in hearts post-MI

As summarized above, GRK2 KO induces a novel Ca^{2+} handling phenotype which maintains a normal myocyte contractility in a way 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 β 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 GRK2 KO mice are potentially due to a combination of increased $I_{Ca,L}$ and normalized NCX activity resulting into reduced SR Ca^{2+} content at baseline. We suspect that the post-MI relatively unchanged and small NCX activity predisposes the GRK2KO MI myocyte to maintain an unchanged SR Ca^{2+} loading 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 GRK2 KO are negated by an LTCC antagonist, verapamil. Elevated SR Ca^{2+} content might also participate in myocyte hypertrophy²⁶. 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 β -adrenergic responsiveness in GRK2 KO myocytes may also contribute to the beneficial effects of GRK2 silencing.

These 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-MI¹⁰. The improvements associated with the loss of GRK2 expression and activity are in a large part attributable to the normalization of intracellular Ca²⁺ 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 Ca²⁺ loading condition and better β -adrenergic responsiveness in unstressed hearts. This enhancement of ECC occurs through differential regulation of sarcolemmal versus SR Ca²⁺ handling, with the net result being improved Ca²⁺ transients leading to the amelioration of the HF phenotype. This is seen at the myocytes 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 Ca²⁺ cycling effected by modulation of I_{Ca,L}. These effects seen at the myocytes level may also contribute to the beneficial effects seen in various HF models treated with the β ARKct peptide as a GRK2 inhibitor^{8, 9, 12}. Further, GRK2 appears to induce novel regulatory modulation on the LTCC since 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Clinical Perspective

GRK2 is a molecular culprit in the development and progression of 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 ECC in unstressed hearts and in failing myocytes. We show that this enhancement of ECC occurs through differential regulation of sarcolemmal versus sarcoplasmic reticulum Ca^{2+} handling, with the net result being improved Ca^{2+} transients without SR Ca^{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^{2+} cycling effected by modulation of the cardiac LTCC. Clear novelty is the compartmentalization of intracellular signaling by GRK2 and thereby differential regulation of Ca^{2+} signaling. Overall, our current results explain the beneficial effects of GRK2 knockdown in cardiac myocytes with intracellular changes in Ca^{2+} cycling being orchestrated to improve overall cardiac-myocyte contractility. Furthermore, our study demonstrates that this interplay between GRK2 and the LTCC might represent an attractive target to correct deranged Ca^{2+} cycling in the failing heart. Future gene therapeutic or pharmacological strategies will thus potentially directly target this interplay and might thus contribute to further improvements in clinical HF treatment.

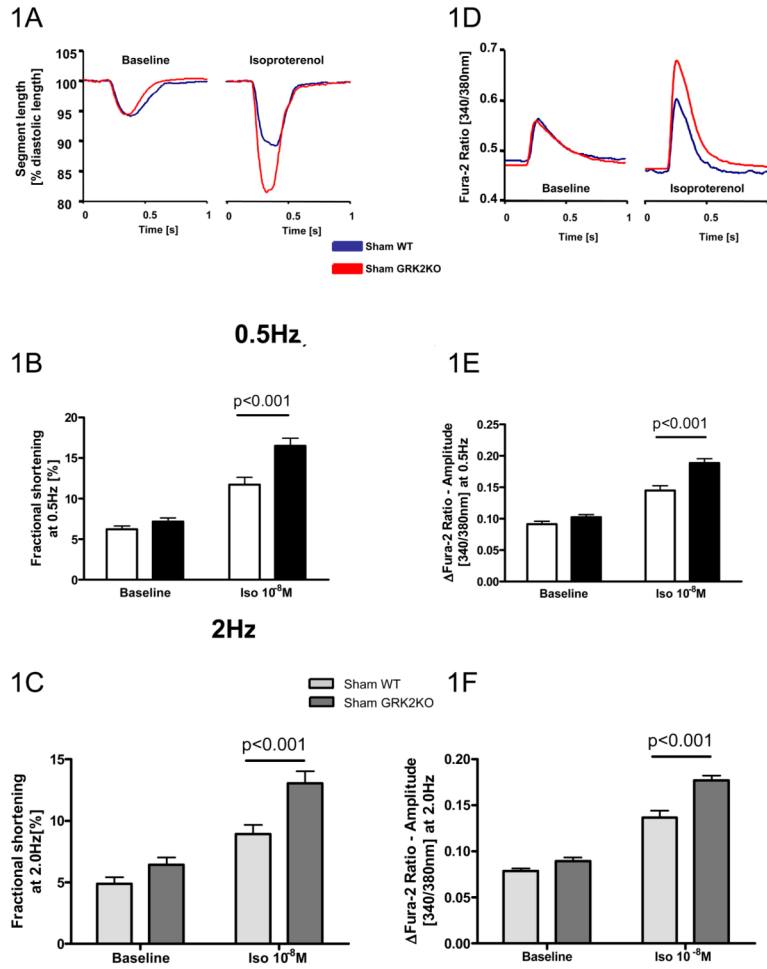


Figure 1.

The loss of GRK2 in myocytes enhances the responsiveness of myocyte contraction and Ca^{2+} transients after β AR 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 [340/380nm 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 48-63 cardiac myocytes from 3 different hearts were measured for each group; two-way ANOVA was used for B, C, E and F.

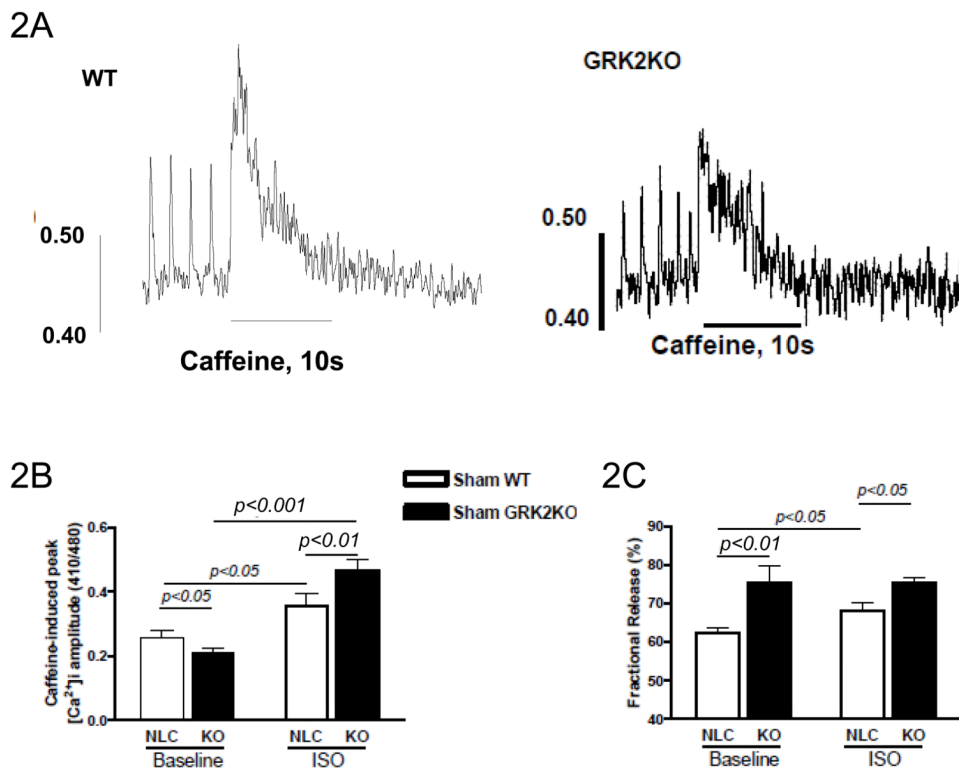
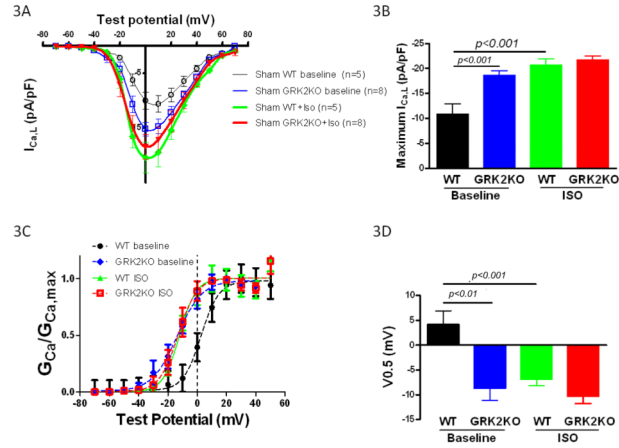


Figure 2. GRK2 silencing in cardiac myocytes alters SR Ca^{2+} content and its regulation. **A**, Representative tracings of the caffeine-induced intracellular Ca^{2+} transients determined with Indo-1 in a control myocyte and a GRK2KO myocyte. **B**, Caffeine-induced peak intracellular Ca^{2+} amplitudes 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-C; a total of 16-29 cardiac myocytes from 3 different hearts were analyzed per group; two-way ANOVA with post hoc student t-test for B and C.

Recording Conditions: 0 Na, 0 K, 2 mM Ca²⁺, 10⁻⁶M isoproterenol



Recording Conditions: 0 Na, 0 K, 2 mM Ca²⁺, 10⁻⁵M H89

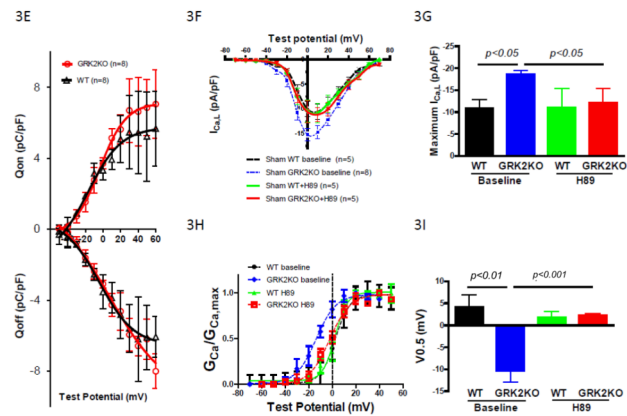


Figure 4

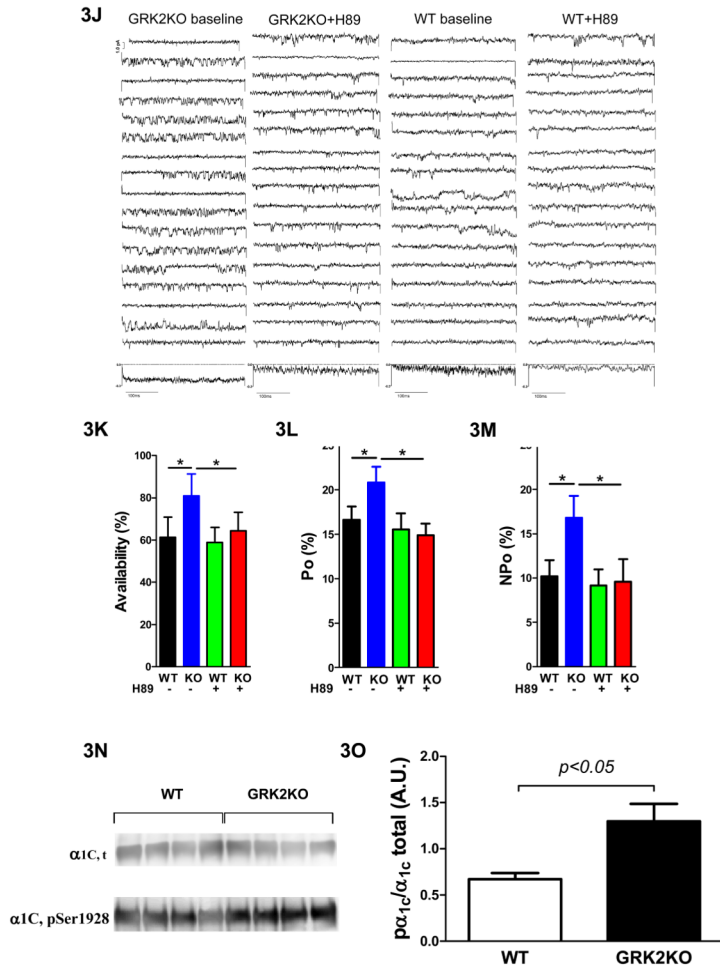
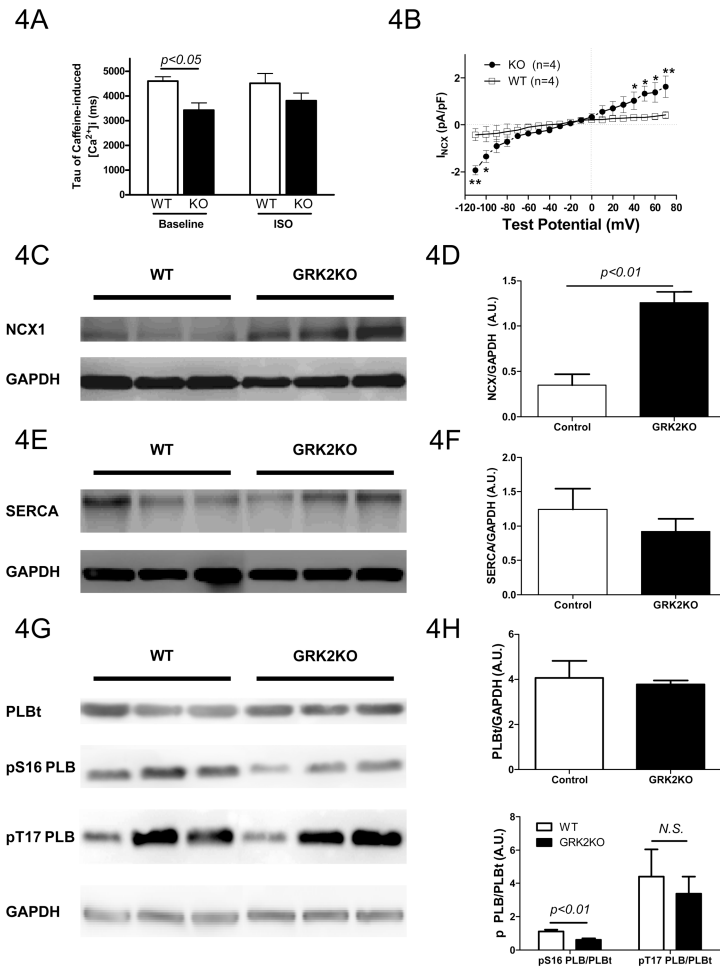


Figure 3.

GRK2 silencing in myocytes increases basal $I_{Ca,L}$ by enhancing PKA-dependent augmentation of LTCC channel activity but reduces its responsiveness to b-adrenergic stimulation. **A**, Current-voltage relationships of $I_{Ca,L}$ (pA/pF) under basal conditions and isoproterenol stimulation. **B**, Peak $I_{Ca,L}$ under basal conditions and isoproterenol stimulation. **C**, Voltage dependence of $I_{Ca,L}$ activation at basal conditions and under isoproterenol stimulation. **D**, Voltage potentials at 50% $I_{Ca,L}$ activation in WT and KO myocytes before and after ISO. **E**, Charge movement measurements in control and KO myocytes at baseline, showing no difference in available LTCCs on the surface membrane in KO and WT myocytes. **F**, The effect of H89 on the current-voltage relationships of $I_{Ca,L}$ in sham WT control and KO myocytes. H89 significantly decreased $I_{Ca,L}$ at most tested voltages in KO myocytes but not in WT myocytes. **G**, Effects of H89 on maximum $I_{Ca,L}$ in WT and KO myocytes. **H & I**, Effects of H89 on $V_{0.5}$ of $I_{Ca,L}$ activation in WT and KO myocytes. **J**, Examples of single channel recordings of the LTCC in KO and WT myocytes before and after the treatment of H89. Sweeps were selected in an increment of 20, i.e., the sweeps shown were sweep 1, 20, 40, etc. The downward deflections were the openings of the channel. At the bottom of the raw sweeps were the ensemble average current of all 400 sweeps recorded. The availability (**K**), open probability in active sweeps (**L**) and the overall open probability (NPo shown in **M**) were significantly increased in KO myocytes but were normalized by H89. H89 did not have significant effects on these parameters of single LTCC activities in WT myocytes. **N**, Western blot of immunoprecipitated total α_{1c} and

phosphorylated a1c at ser1928. **O**, Phosphorylation level of a1c in unstressed WT and GRK2KO hearts. n=5 animals/group, two-way ANOVA with post hoc t-test was used for comparison for A-M in this figure; t-test was used for O.



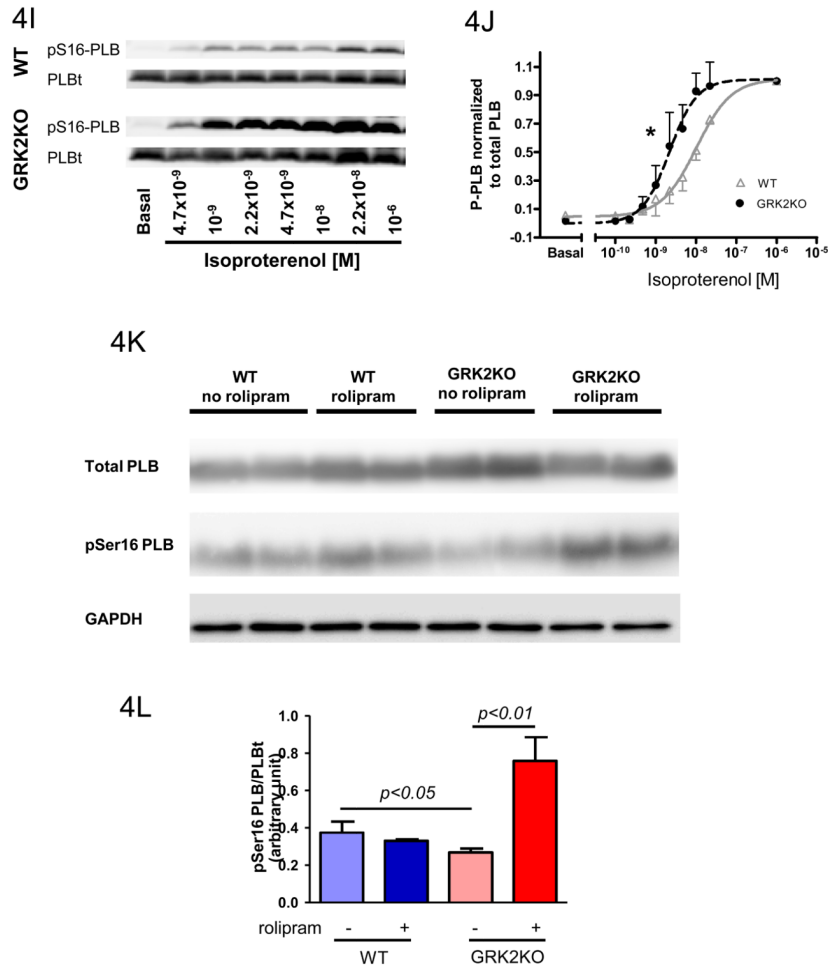


Figure 4. GRK2 silencing in cardiac myocytes increased NCX expression, reduced PLB phosphorylation by PKA dependent PDE4 activation and enhanced responsiveness to ISO. **A**, Tau values of caffeine-induced intracellular Ca²⁺ transient decay, indicating increased NCX activity in GRK2KO myocytes; n=16-29 cardiac myocytes from 3 different hearts. **B**, NCX currents at baseline in control (WT) and GRK2KO myocytes; n=4 animals/group, two-way repeated ANOVA with post-hoc 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 3-5 hearts were analyzed for each group; one-way ANOVA for **L**.

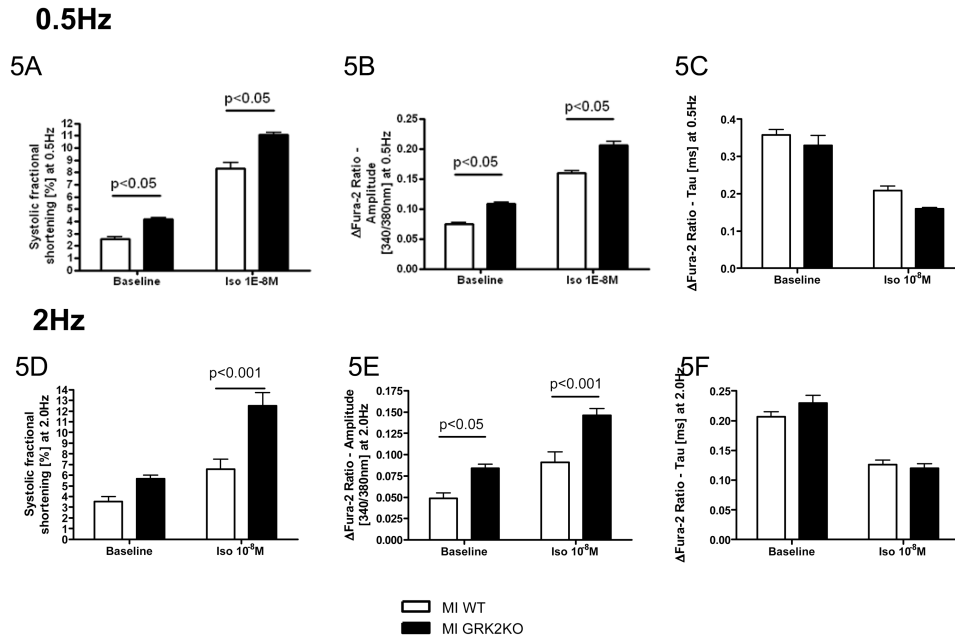


Figure 5. Loss of GRK2 in cardiac myocytes ameliorates single cell contractility and Ca²⁺ handling post-MI. Measurements were obtained at 0.5Hz (**A-C**) or 2Hz (**D-F**) under basal conditions and isoproterenol stimulation (10⁻⁸M). Fractional shortenings (**A & D**), Fura-2 ratio amplitudes (**B & E**) representing the change of intracellular Ca²⁺ transient from baseline, and tau of Ca²⁺ transient decay (**C & F**) were shown. For all measurements a total of 60 cardiac myocytes from 6 different hearts were measured for each group; two-way ANOVA.

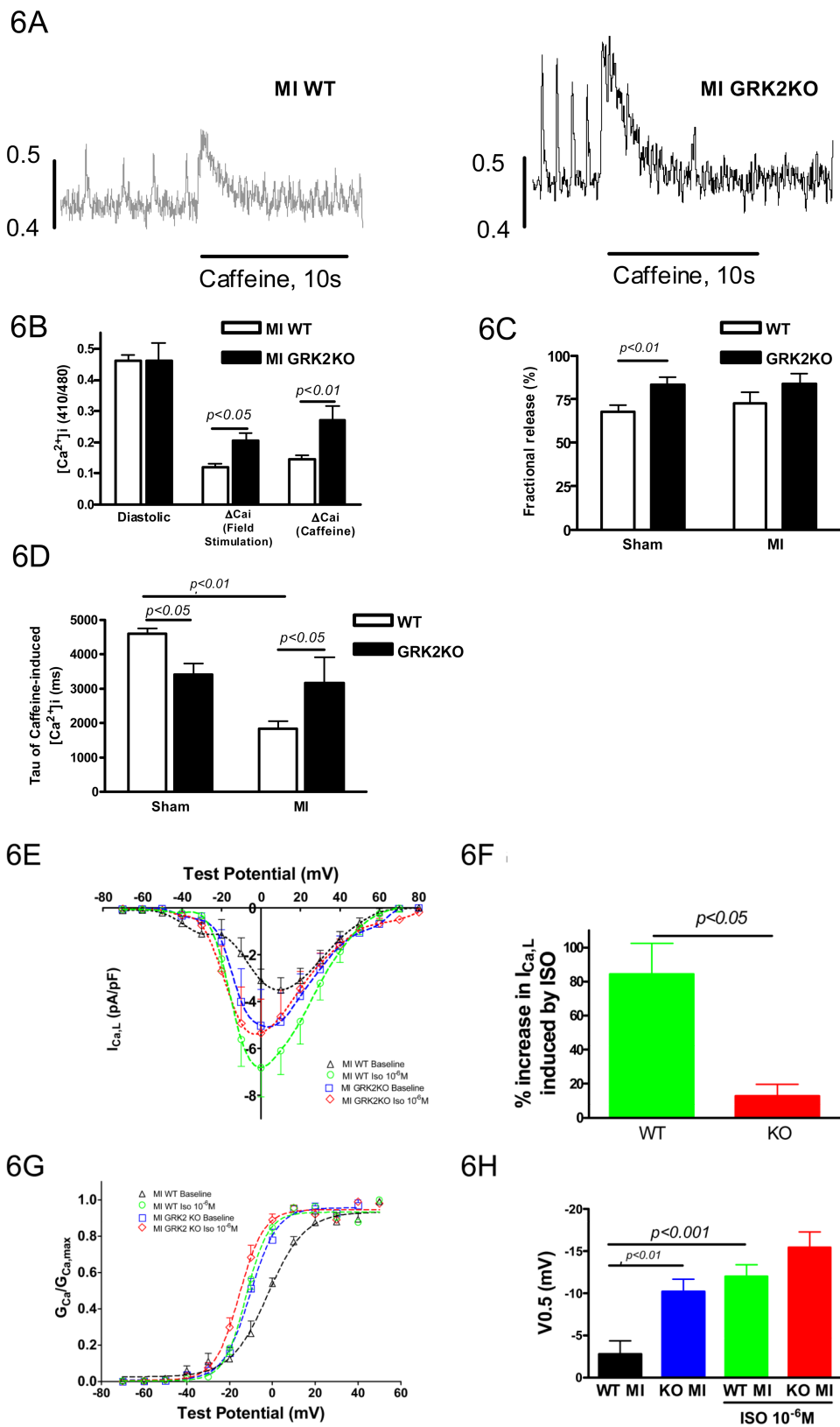


Figure 6.

Myocyte GRK2 silencing inhibits adverse Ca^{2+} handling remodeling post-MI. **A**, Representative tracings of the caffeine-induced intracellular Ca^{2+} transients determined with Indo-1; **B**, Amplitudes of Ca^{2+} transients elicited by field stimulation (0.5Hz) and caffeine-spritz; **C**, Fractional release calculated as the ratio of peak Ca^{2+} concentration induced by field stimulation to peak Ca^{2+} concentration induced by caffeine; **D**, Tau of caffeine-induced intracellular Ca^{2+} transients. n=14-23 cells from 4-5 animals/group, unpaired two-tailed t-test for B, two-way ANOVA for C and D; **E**, Current-voltage relationship of $I_{\text{Ca,L}}$ (pA/pF) under basal conditions and isoproterenol stimulation in post-MI WT and KO myocytes; **F**, % increase in peak $I_{\text{Ca,L}}$ by isoproterenol. **G**, Voltage dependence (G-V curve) of $I_{\text{Ca,L}}$ activation at basal conditions and under isoproterenol stimulation. **H**, Voltages at 50% $I_{\text{Ca,L}}$ activation; n=7 cells from 3-4 different animals/group; one-way ANOVA for E-H.

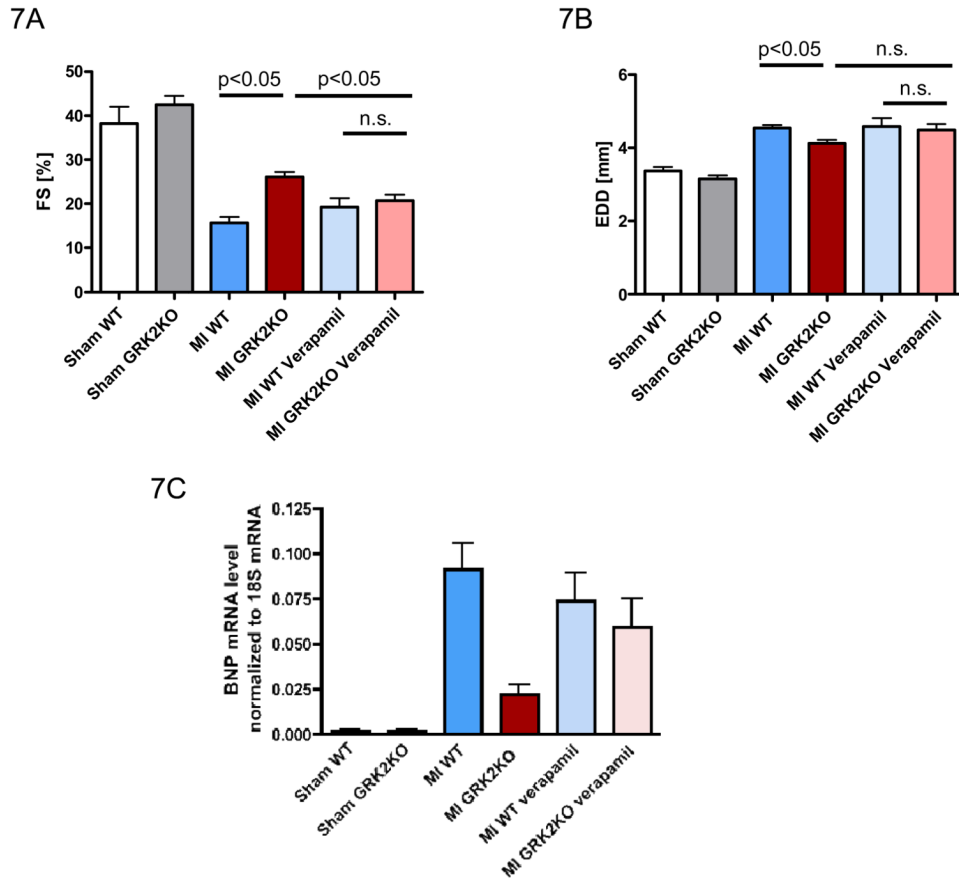


Figure 7. Beneficial effects of myocytes GRK2 lowering are reduced by the LTCC blocker verapamil. Echocardiographic measurements of fractional shortening [FS] (A) and left ventricular end diastolic diameter [EDD] (B) at 42 days post MI or sham operation. Mice were supplemented orally with verapamil starting 14 days post MI until the end of the study period (= 42 days post MI). n=6-8/sham group, n=15-25/MI group without verapamil, n=10-13/MI group with verapamil, one-way ANOVA. C, cardiac myocyte BNP mRNA levels 42 days post MI or sham operation; n=5-6/group for sham, n=8-9/group for MI; one-way ANOVA and unpaired two-tailed t-test.