SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Experimental animals

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

Experimental protocols

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

Isolation of cardiac myocytes and maintenance of primary cultures

Adult mouse cardiac myocytes were isolated as previously described[5]. For Ca^{2+} transients and single myocyte contractility measurements, cells were used within 8 hours after isolation. For in vitro cell culture assays, freshly isolated cells were washed and resuspended with α-MEM (Gibco, Invitrogen Corporation, Carlsbad, California, USA) supplemented with Hanks' salt, and then plated onto laminin (Invitrogen Corporation, Carlsbad, California, USA) pre-coated culture dishes. Cells were maintained in α-MEM with Hank's salt solution supplemented with L-Glutamine (10 mmol/l), 2, 3-Butandionemonoxime (BDM, 10mmol/l)

(Sigma-Aldrich, St. Louis, Missouri, USA) and Insulin-Transferrin-Selenium (1:1000 dilution) (Gibco, Invitrogen Corporation, Carlsbad, California, USA) with a humidified atmosphere containing 2% 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 [9]: $[Ca^{2+}]_i=Kd*R/((Kd/[Ca^{2+}]_{rest})+1-R)$, where R is the ratio of emitted fluorescence to the resting emitting fluorescence. $[Ca^{2+}]_{rest}$ was considered as 100nM and Kd was 1100nM.The ratio of peak Ca^{2+} concentration induced by stimulation to peak Ca^{2+} concentration induced by caffeine was used as the index of fractional release.

Electrophysiology

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

Single L-type Ca2+ channel currents were recorded in cell-attached patches with an Axopatch 200B amplifier and Clampex 10. The holding potential was -90mV assuming the intracellular resting membrane potential was about -70mV (the averaged resting membrane potentials in control and DTG myocytes during electrophysiological measurements) and depolarized to 10mV. The recording glass pipette contained (in mM): $BaCl_2$, 70; Sucrose, 70; NMDG, 10; HEPES, 10; TEA-Cl (tetraammonium chloride), 10; tetrodotoxin (TTX), 0.05; and 4-Aminopyridine (4-AP), 5; pH7.4 with TEA-OH. The bath solution was normal Tyrode solution containing (in mM): NaCl 150, KCl 5.4, $MgCl_2$ 1.2, HEPES 5, Glucose 10, Na-pyruvate 2, $CaCl_2$ 1, pH to 7.4 with NaOH. After 400 continuous sweeps of recording at 0.5Hz, the external solution was changed to a H89 (5μM) containing bath solution and 15 minutes were waited to allow H89 to fully take effect. Then, another 400 continuous sweeps of recording were made. Single-channel and whole-cell Ca^{2+} currents were analyzed with Clampfit 10. To quantify the amount of LTCCs on the surface membrane of the myocytes, charege movement measurements of the LTCC were done with pCLAMP10 and an Axon 200B amplifier as described previously[10]. Only myocytes with minimal (<10%) rundown of $I_{Ca,L}$ were included in the data sets. $I_{Ca,L}$ was measured at baseline and under stimulation with 10^{-6}M Isoproterenol. To study the effect of H89 (a PKA inhibitor, Sigma-Aldrich, 5μM) on $I_{Ca,L}$, 5μM H89 was included with the pipette solution for 10 minutes to allow adequate diffusion of H89 into the cell to inhibit PKA.

Sodium-calcium exchange current (INCX) was recorded as described previously[11]. The myocyte was bathed in a K^+-free solution containing (in mmol/L): NaCl 145, $MgCl_2$ 1, HEPES 5, $CaCl_2$ 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_2ATP 5, $CaCl_2$ 6, $MgCl_2$ 4, HEPES 10, TEA-Cl 20, EGTA 21, ryanodine 0.0005, pH 7.2 with CsOH. The cell was dialyzed for 10 minutes after rupturing the patch and the membrane current was recorded with a ramp test (+80mV to -80mV at 100mV/s) following a 100-ms depolarization to +80 from the holding potential of -40mV. During the recording, the bath solution then was changed to the K^+-free solution with 5mmol/L Ni^{2+}. Recording was stopped once a stable effect of Ni^{2+} was seen and the Ni^{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 [2]. cDNA was synthesized and expression levels of NCX and BNP were analyzed using quantitative Real-Time PCR[2]; 28S or 18S mRNA levels were used for normalization.

Western blot analysis

Western blotting was performed as described previously [3]. Cardiac protein levels of GRK2 (sc-562, C-15, Santa Cruz Biotechnology, 1:5,000), SERCA (Clone II-H-11, Sigma-Aldrich, St. Louis, Missouri, USA), PLB, pPLB S16, pPLB Thr17 (A010-14, -12, and -13, Badrilla, Leeds, United Kingdom), and GAPDH (clone 6C5, Millipore, Billerica, Massachusetts, USA) were assessed in cardiac myocyte cellular preparations. Protein content was quantified with the BioRad DC Protein Assay (BioRad Laboratories, Richmond, California, USA). Protein samples were separated by 4-20% SDS-PAGE (Invitrogen Corporation, Carlsbad, California, USA), and proteins were transferred to PVDF membrane (Millipore Corporation, Billerica, Massachusetts, USA) and probed with the first antibody at 4°C overnight. The proteins were

stained with a corresponding Alexa Fluor 680- (Molecular Probes; 1:10.000) or IRDye 800CW-coupled (Rockland Inc.; 1:10.000) secondary antibody, followed by visualization of the proteins with a LI-COR infrared imager (Odyssey, LI-COR, Lincoln, Nebraska, USA), and quantitative densitometric analysis was performed applying Odyssey version 2.0 infrared imaging software. α1c immunoprecipitation was performed with the Dynabeads Protein A for Immunoprecipitation Kit (Invitrogen Corporation, Carlsbad, California, USA) with 700μg crude protein extracted from each frozen heart according to the manufacture's instruction. The antibody against α1c for immunoprecipitation was purchased from Millipore (Billerica, MA, USA) and the antibodies for the detection of α1c and p1928 α1c were from NeuroMab (Davis, CA, USA).

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

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

Echocardiographic analysis of cardiac function

Two-dimensional transthoracic echocardiography was acquired with a 12-MHz probe and a Vevo770 imaging system (VisualSonics Inc., Toronto, Ontario, Canada) both in sham and infarcted mice as described in details elsewhere [3]. 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

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

Data supplement table 1: Echocardiography for determination of cardiac function in WT and GRK2KO mice with (MI) or without MI (Sham). In vivo cardiac function was assessed by echocardiography twenty-eight days after MI. n=10animals/sham group, n=15-19animals/MI group, *: p<0.05.

	Sham WT	Sham GRK2KO	MI WT	MI GRK2KO
FS at 0.5Hz [%]	6.24±0.42	7.16±0.44	2.56±0.19#	4.19±0.14%
$[Ca^{2+}]_i$ peak at 0.5Hz [Fura-2,340/380]	0.091±0.005	0.103±0.004	0.075±0.002#	0.108±0.003%
FS at 2Hz [%]	4.90±0.51	6.43±0.58	3.53±0.48	5.65±0.35
$[Ca^{2+}]_i$ peak at 2Hz [Fura-2, 340/380]	0,078±0.003	0.089±0.004	0.048±0.006#	0.084±0.005%
SR Ca^{2+} load [indo-1, 410/480]	0.26±0.02	0.22±0.01*	0.15±0.01#	0.26±0.04%
Fractional Ca^{2+} release [%]	64.4±1.3	75.4±4.4*	70.6±6.1	83.9±5.9%
I_{Ca-L} peak [pA/pF]	-10.8±2.0	-18.57±0.9*	-3.5±1.3#	-5.0±1.5$%

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 Ca^{2+} amplitudes under baseline conditions (SR Ca2+ load [indo-1, 410/480]); fractional release calculated as the ratio of peak Ca^{2+} concentration induced by field stimulation to peak Ca^{2+} 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

1. Matkovich SJ, Diwan A, Klanke JL, Hammer DJ, Marreez Y, Odley AM, Brunskill EW, Koch WJ, Schwartz RJ, Dorn GW, 2nd. Cardiac-specific ablation of g-protein receptor kinase 2 redefines its roles in heart development and beta-adrenergic signaling. *Circ Res*. 2006;99:996-1003
2. Raake PW, Vinge LE, Gao E, Boucher M, Rengo G, Chen X, DeGeorge BR, Jr., Matkovich S, Houser SR, Most P, Eckhart AD, Dorn GW, 2nd, Koch WJ. G protein-coupled receptor kinase 2 ablation in cardiac myocytes before or after myocardial infarction prevents heart failure. *Circ Res*. 2008;103:413-422
3. Most P, Seifert H, Gao E, Funakoshi H, Volkers M, Heierhorst J, Remppis A, Pleger ST, DeGeorge BR, Jr., Eckhart AD, Feldman AM, Koch WJ. Cardiac s100a1 protein levels determine contractile performance and propensity toward heart failure after myocardial infarction. *Circulation*. 2006;114:1258-1268
4. Cohn RD, Durbeej M, Moore SA, Coral-Vazquez R, Prouty S, Campbell KP. Prevention of cardiomyopathy in mouse models lacking the smooth muscle sarcoglycan-sarcospan complex. *J Clin Invest*. 2001;107:R1-7
5. Zhou YY, Wang SQ, Zhu WZ, Chruscinski A, Kobilka BK, Ziman B, Wang S, Lakatta EG, Cheng H, Xiao RP. Culture and adenoviral infection of adult mouse cardiac myocytes: Methods for cellular genetic physiology. *Am J Physiol Heart Circ Physiol*. 2000;279:H429-436
6. Tocchetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Di Benedetto G, O'Rourke B, Gao WD, Wink DA, Toscano JP, Zaccolo M, Bers DM, Valdivia HH, Cheng H, Kass DA, Paolocci N. Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum ca2+ cycling. *Circ Res*. 2007;100:96-104
7. Wei SK, Ruknudin A, Hanlon SU, McCurley JM, Schulze DH, Haigney MC. Protein kinase a hyperphosphorylation increases basal current but decreases beta-adrenergic responsiveness of the sarcolemmal na+-ca2+ exchanger in failing pig myocytes. *Circulation research*. 2003;92:897-903
8. Terracciano CM, Souza AI, Philipson KD, MacLeod KT. Na+-ca2+ exchange and sarcoplasmic reticular ca2+ regulation in ventricular myocytes from transgenic mice overexpressing the na+-ca2+ exchanger. *J Physiol*. 1998;512 (Pt 3):651-667
9. Ginsburg KS, Bers DM. Modulation of excitation-contraction coupling by isoproterenol in cardiomyocytes with controlled sr ca2+ load and ca2+ current trigger. *J Physiol*. 2004;556:463-480
10. Chen X, 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
11. Nakayama H, Chen X, Baines CP, Klevitsky R, Zhang X, Zhang H, Jaleel N, Chua BH, Hewett TE, Robbins J, Houser SR, Molkentin JD. Ca2+- and mitochondrial-dependent cardiomyocyte necrosis as a primary mediator of heart failure. *J Clin Invest*. 2007;117:2431-2444