Phosphodiesterase 2 Protects Against Catecholamine-Induced Arrhythmia and Preserves Contractile Function After Myocardial Infarction

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**Rationale:** Phosphodiesterase 2 is a dual substrate esterase, which has the unique property to be stimulated by cGMP, but primarily hydrolyzes cAMP. Myocardial phosphodiesterase 2 is upregulated in human heart failure, but its role in the heart is unknown.

**Objective:** To explore the role of phosphodiesterase 2 in cardiac function, propensity to arrhythmia, and myocardial infarction.

**Methods and Results:** Pharmacological inhibition of phosphodiesterase 2 (BAY 60–7550, BAY) led to a significant positive chronotropic effect on top of maximal β-adrenoceptor activation in healthy mice. Under pathological conditions induced by chronic catecholamine infusions, BAY reversed both the attenuated β-adrenoceptor-mediated inotropy and chronotropy. Conversely, ECG telemetry in heart-specific phosphodiesterase 2 transgenic (TG) mice showed a marked reduction in resting and in maximal heart rate, whereas cardiac output was completely preserved because of greater cardiac contraction. This well-tolerated phenotype persisted in elderly TG with no indications of cardiac pathology or premature death. During arrhythmia provocation induced by catecholamine injections, TG animals were resistant to triggered ventricular arrhythmias. Accordingly, Ca2+-spark analysis in isolated TG cardiomyocytes revealed remarkably reduced Ca2+ leakage and lower basal phosphorylation levels of Ca2+-cycling proteins including ryanodine receptor type 2. Moreover, TG demonstrated improved cardiac function after myocardial infarction.

**Conclusions:** Endogenous phosphodiesterase 2 contributes to heart rate regulation. Greater phosphodiesterase 2 abundance protects against arrhythmias and improves contraction force after severe ischemic insult. Activating myocardial phosphodiesterase 2 may, thus, represent a novel intracellular antiadrenergic therapeutic strategy protecting the heart from arrhythmia and contractile dysfunction. (Circ Res. 2017;120:120-132. DOI: 10.1161/CIRCRESAHA.116.310069.)

**Key Words:** cardiac arrhythmia ■ catecholamine ■ cyclic GMP-stimulated phosphodiesterase ■ heart rate ■ myocardial contraction
Heart failure (HF) is among the most common causes of morbidity and mortality worldwide. A characteristic pathophysiological feature of HF is the chronic activation of the sympathetic nervous system. Although initially aimed to maintain cardiac output, constant stimulation of β-adrenoceptors (β-ARs) results in molecular and structural changes, such as hypertrophy, cardiac fibrosis, and electromechanical dysfunction. This process creates a setting for lethal cardiac arrhythmias, which may account for ≈40% of deaths in patients with HF. Moreover, increased resting heart rate and lower heart rate variability are significant prognostic risk factors for mortality and cardiovascular outcome. 

The sympathetically stressed heart responds with desensitization mechanisms, which involve the reduction of functional β-AR and the redistribution of β-AR at the plasma membrane, but also a modulation in abundance (the necessary dose to substantially improve prognosis). 

Accordingly, pharmacological blockade of receptor activation could represent a novel therapeutic approach in heart disease.

In the present study, we show that phosphodiesterase 2 tonically reduces heart rate and controls both β-AR chronotropic and inotropic responsiveness under stressed conditions in vivo. To evaluate short- and long-term effects of chronically increased phosphodiesterase 2 activity in the heart, we generated a cardiac phosphodiesterase 2-transgenic (TG) mouse line. Our study reveals that greater phosphodiesterase 2 abundance lowers heart rate without impairment of cardiac contractility in vivo and prevents ventricular arrhythmias by preventing Ca++ leakage from the sarcoplasmic reticulum (SR). In experimental myocardial infarction (MI), higher phosphodiesterase 2 abundance improved ventricular function. Consequently, phosphodiesterase 2 activation could represent a novel therapeutic approach in heart disease.

Methods
For a detailed description of methods including surgical procedures, see Online Data Supplement.

Chronic Isoproterenol Administration
Isoproterenol (30 μg/g/d; Sigma-Aldrich) was delivered to mice by subcutaneously implanted osmotic minipumps (Alzet; model 2002).

Application of Dobutamine, BAY 60–7550, and Metoprolol for Echocardiographic Experiments
Anesthetized mice were analyzed by echocardiography first under basal conditions and then 5 minutes after the injection of dobutamine (IP, 10 mg/kg). When indicated, BAY (IP, 3 mg/kg) was applied 10 minutes post dobutamine injection, and after an additional period of 10 minutes, cardiac function was again echocardiographically monitored. For metoprolol studies, doses from 1 to 100 mg/kg (IP) were cumulatively applied with 10-minute intervals between injections and echocardiographic measurements.

Generation of Phosphodiesterase 2-TG Mice
Phosphodiesterase 2-TG mice were generated by using a plasmid containing the murine sequence of the splice variant phosphodiesterase 2A3 (NM_001008548.3). Expression was set under the control of the human α-myosin heavy chain promoter to ensure cardiac specificity. Transgenesis was achieved by pronuclear injection of linearized plasmids into isolated zygotes of a FVB/N donor strain. Successful transformation of the offspring was assessed by polymerase chain reaction, and overexpression levels were determined by immunoblot analysis. Resulting founder lines were crossed into a C57Bl/6 background.

ECG Telemetry Recordings and Arrhythmia Provocation
ECGs were recorded in freely moving unrestrained mice. Arrhythmia provocation was performed by double injections of isoproterenol (IP 2 mg/kg) separated by an interval of 30 minutes; analysis was performed for 90 minutes after the first injection.
Isolation of Adult Mouse Ventricular Myocytes
Adult mouse ventricular myocytes were isolated by Langendorff perfusion as described previously.11

CAMP Measurements by Förster Resonance Energy Transfer
Adult mouse ventricular myocytes were infected with an adenovirus encoding the Epac-S65T cAMP Förster Resonance Energy Transfer (FRET) probe for 24 hours.32 Changes in cAMP levels were assessed by YFP/CFP (yellow fluorescent protein/cyan fluorescent protein) emission ratios.

Patch-Clamp Studies
I_{Ca,L} was recorded in the whole-cell configuration of the patch-clamp technique.33

Ca2+ Spark Analysis
Ca2+ spark measurements were performed on a laser scanning confocal microscope (LSM 5 Pascal, Zeiss). Fluorescence images of Fluo-3 AM (10 μmol/L; Molecular Probes)–loaded ventricular myocytes were recorded in the line-scan mode.

Measurement of Ca2+ Transients, Sarcomere Shortening, SR Ca2+ Leak, and Load
Isolated mouse ventricular cardiomyocytes were loaded with 3 μmol/L Fura-2 AM (Invitrogen). Sarcomere shortening was simultaneously recorded with Fura-2 ratios (IonOptix) as described previously.33 Myocytes were electrically stimulated at a frequency of 0.5 Hz.

Immunoblot Analysis
Protein samples were prepared from pulverized ventricular myocardium and lysed in a buffer containing 30 mmol/L Tris/HCl (pH 8.8), 5 mmol/L EDTA, 30 mmol/L NaF, 3% SDS, and 10% glycerol. Samples were separated in denaturing acrylamide gels and subsequently transferred onto nitrocellulose or PVDF membranes. After blocking the membranes with Roti-block (Carl Roth) for 1 hour, the incubation with anti-calsequestrin (1:1000; ThermoScientific), anti-SERCA2a (sarco/endoplasmic reticulum Ca2+-ATPase 2a), anti-phosphodieserine 2 (each 1:200; Santa Cruz), anti-pPLB-S16, anti-pPLB-T17, and anti-β-adrenoceptor (β-AR)–induced inotropy in animals chronically treated with isoproterenol (ISO). Effect of PDE2 inhibition in mice exposed to chronic ISO infusions for 7 days (30 mg/kg/d) or NaCl (0.9%) as control. As expected, isoproterenol-treated animals developed prominent cardiac hypertrophy, indicated by an increase in left ventricular weight to body weight ratio from 3.6±0.2 to 4.7±0.1 mg/g (P<0.05; Online Figure I). After chronic isoproterenol treatment, the positive chronotropic and inotropic effects of dobutamine were abrogated, indicating desensitization of the β-ARs (Figure 1). This was completely reversed by inhibition of phosphodiesterase 2 with BAY 60–755034 (BAY, 3 mg/kg), restoring β-AR responsiveness to the level observed in the control group (Figure 1). Interestingly, phosphodiesterase 2 inhibition also had an effect on control mice, almost doubling the impact of β-AR stimulation on heart rate over the average basal heart rate of 42±18 bpm from 95±29 to 170±23 bpm (Figure 1B). The dosage of 3 mg/kg was chosen according to recent publications on in vivo experiments in which phosphodiesterase 2 inhibition also had an effect on control mice.
rodents and unpublished pharmacokinetic studies provided by BAYER. This restriction of phosphodiesterase 2 to chronotropic regulation under physiological conditions was supported by a study on beagle dogs treated with increasing doses of BAY (3, 10, and 30 mg/kg). In line with the murine model, the inhibition of phosphodiesterase 2 predominantly resulted in acceleration of heart rate (10 mg/kg: +20%; 30 mg/kg: +28%; Online Figure IIA), whereas stroke volume, cardiac output, and systolic and diastolic blood pressures remained largely unchanged (Online Figure IIB). Taken together, the role of phosphodiesterase 2 seems restricted to heart rate regulation under physiological conditions, whereas its stress-induced upregulation contributes to the desensitization of both β-AR–induced increases in heart rate and contraction force.

**Effect of Phosphodiesterase 2 Overexpression on Heart Rate and Cardiac Function**

To gain insight into the consequences of higher cardiac phosphodiesterase 2 levels, we generated TG mouse lines that overexpress phosphodiesterase 2 ≈6- to 15-fold specifically in cardiomyocytes (Figure 2H; Online Figures III and IV). The low-expressing (6-fold) line TG-4808 did not show any overt phenotype (Online Figure IIA), whereas its stress-induced upregulation contributes to the desensitization of both β-AR–induced increases in heart rate and contraction force.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Higher basal contractility and lower heart rate in phosphodiesterase 2 (PDE2)-transgenic (TG) mice. Echocardiographic determination of fractional area shortening (FAS, A), heart rate (HR, B), and cardiac output (CO, C) in anesthetized 2-mo-old mice. Animals were treated with 10 mg/kg dobutamine (DOBU, IP) 2 min before measurements when indicated; n=7 to 9 for each group. Effect of metoprolol (100 mg/kg, IP) on (D) FAS and (E) HR in anesthetized animals. F. Correlation between the reduction of FAS and HR in the presence of increasing metoprolol doses (METO 0, 1, 3, 10, 30, and 100 mg/kg); n=5 for each group. G. Left ventricular weight (LVW) calculated from the echocardiographic data and normalized to tibia length. H. Lysates prepared from ventricular myocardium were analyzed by immunoblot with the indicated specific antibodies. PDE2 expression was normalized to calsequestrin (CSQ) and given relative to WT; n=7 to 9 for each group. Statistical significance was determined by 1-way ANOVA followed by Newman–Keuls multiple comparison test (A, B, D, and E) and Student t test to compare the 2 genotypes on basal level (A–E, G, and H). *P<0.05 vs WT, #P<0.05 vs respective basal.
displayed a substantial lower basal and dobutamine-stimulated maximal heart rate compared with wild type (WT) with an average difference of 77±17 and 98±14 bpm, respectively (Figure 2B). Basal contraction force measured as fractional area shortening was higher in TG-4320 than in WT (37±1% versus 32±1%), whereas maximal dobutamine-stimulated contractility remained unaffected (80±3% in WT and 77±2% in phosphodiesterase 2-TG; Figure 2A). In line with this, the lower heart rate combined with higher basal contraction produced a cardiac output virtually identical to that of WT controls (Figure 2C). The specific phenotype of lower HR shortening 31±2% in WT versus 44±2% in phosphodiesterase 2-TG demonstrated by a rightward shift of the Gaussian distribution curve (Figure 4C). The difference in RR pattern was further confirmed by Poincaré analysis (Online Figure VICA and VID). Notably, the broadening of the RR distribution curve (Figure 4C) indicated higher heart rate variability in phosphodiesterase 2-TG compared with WT animals (Figure 4D). Thus, overexpression of phosphodiesterase 2 recapitulates the classical shift in sympathetic/parasympathetic balance as observed during an increased parasympathetic control of heart rate regulation.37 However, phosphodiesterase 2-TG displayed the same relation of heart rate and physical activity as WT littermates, indicating preserved chronotropic competence (Figure 4E). Basal heart rate reduction with maintained autonomic control has been allocated to a decrease in hyperpolarization-activated cyclic nucleotide-gated (HCN) channel activity.38 Therefore, we investigated how phosphodiesterase 2 action, we investigated how phosphodiesterase 2 overexpression impacts cAMP levels and Ca2+ cycling.

First, we analyzed the effects of phosphodiesterase 2 overexpression on cardiac morphology and performance throughout most of the animals’ life span including elderly mice. Notably, during 18 months of serial echocardiography, low heart rate and higher cardiac performance were preserved with no indication of functional decline, maladaptive remodeling, or premature death (Figure 3). The prominent impact on basal heart rate prompted us to investigate the effect of phosphodiesterase 2 on heart rate regulation by telemetric ECG recordings in unrestrained, freely moving mice. Circadian analysis over the course of 24 hours confirmed significantly lower heart rates in phosphodiesterase 2-TG animals during low activity daytime (105±17 bpm) and high activity nighttime (90±24 bpm; Figure 4A and 4B; Online Figure VICA and VID). Accordingly, analysis of the respective RR intervals recorded for 24 hours showed longer average intervals in phosphodiesterase 2-TG demonstrated by a rightward shift of the Gaussian distribution curve (Figure 4C). The difference in RR pattern was further confirmed by Poincaré analysis (Online Figure VICA and VID). Notably, the broadening of the RR distribution curve (Figure 4C) indicated higher heart rate variability in phosphodiesterase 2-TG compared with WT animals (Figure 4D). Thus, overexpression of phosphodiesterase 2 recapitulates the classical shift in sympathetic/parasympathetic balance as observed during an increased parasympathetic control of heart rate regulation.37 However, phosphodiesterase 2-TG displayed the same relation of heart rate and physical activity as WT littermates, indicating preserved chronotropic competence (Figure 4E). Basal heart rate reduction with maintained autonomic control has been allocated to a decrease in hyperpolarization-activated cyclic nucleotide-gated (HCN) channel activity.38 Therefore, we investigated the impact of the HCN-blocker ivabradine (IP 5 µg/g) on heart rate (Figure 4F; Online Figure VIE). WT and TG animals showed similar susceptibility to ivabradine treatment displaying no significant difference in either lowest heart rate (348±23 and 319±17 bpm, respectively) or average heart rate reduction (158±10 bpm for WT and 128±14 bpm for TG).
overexpression affects intracellular cAMP levels. For that, we assessed real-time changes in β-AR–induced intracellular cAMP levels by FRET measurements in isolated ventricular myocytes infected with an adenovirus expressing the FRET-based cAMP probe Epac-SH187.32 The response to a 15-second application of isoproterenol (30 nmol/L) was markedly blunted in phosphodiesterase 2-TG myocytes, which showed an average change over basal of 17% compared with an average of 120% displayed by WT animals (Figure 5A).

Next, we analyzed the consequences of the reduced accumulation of intracellular cAMP levels on cellular Ca²⁺ handling in phosphodiesterase 2-TG mice. In line with previous publications, phosphodiesterase 2 overexpression markedly attenuated the β-AR–induced increase in ICa,L from 35% in WT to 8% in phosphodiesterase 2-TG (Figure 5B), whereas basal ICa,L amplitude remained unaffected (data not shown). This effect was fully reversed by phosphodiesterase 2 inhibition with BAY (Figure 5C). Accordingly, Ca²⁺ transient and contractility analysis in field-stimulated isolated ventricular myocytes revealed an attenuation of the β-AR response to isoproterenol, whereas basal contractility and Ca²⁺ transients were similar between WT and phosphodiesterase 2-TG mice (Online Figure VII). SR Ca²⁺ load and fractional release were evaluated during the SR leak protocol (see below) by rapid application of 10 mmol/L caffeine. As expected, SR Ca²⁺ load and the fraction of released Ca²⁺ from the SR during systole significantly increased in WT-derived ventricular myocytes after β-AR stimulation. In contrast, the isoproterenol-induced increase in fractional SR Ca²⁺ release was absent in phosphodiesterase 2-TG, whereas SR Ca²⁺ content was not affected neither under basal nor under stimulated conditions (Figure 5D and 5E). This observation is in line with the lower β-AR response of systolic Ca²⁺ amplitude and force development measured in myocytes from phosphodiesterase 2-TG (Online Figure VII).

Arrhythmia Provocation
To test the effect of greater phosphodiesterase 2 abundance under acute β-AR stress, animals received 2 injections of isoproterenol (2 mg/kg) separated by an interval of 30 minutes.39 As expected from echocardiographic analysis (Figure 2B) and heart rate/activity correlation (Figure 4E), phosphodiesterase 2-TG displayed a lower maximal heart rate on β-AR stimulation (693±9 bpm in WT versus 610±5 bpm in phosphodiesterase 2-TG), whereas chronotropic adaptation, ie, absolute increase
over basal heart rate, was maintained (157±18 bpm and 154±11 bpm, respectively; Figure 6A and 6B). ECG was monitored for arrhythmic events such as ventricular extra systoles, salvos, and ventricular tachycardia (VT) during a period of 90 minutes after the first injection. All animals tested developed ventricular extra systoles with frequency of occurrence increasing ≈15 minutes after the second injection of isoproterenol (Online Figure VIII A). The cells were depolarized every 8 s from ~50 to 0 mV during 400 ms; n=3 animals/genotype with 10 to 19 cell in each group. D, Mean amplitude of SR Ca²⁺ load in the presence or absence of ISO measured as the change in Fura-2 ratio after rapid application of caffeine (10 mmol/L). E and F, SR Ca²⁺ load and fractional release measured in Fura-2–loaded adult mouse ventricular myocytes paced at 1 Hz, in the presence or absence of ISO (100 nmol/L). E, Mean fractional Ca²⁺ release in control or ISO calculated as the ratio of Ca²⁺ transient amplitude divided by caffeine-induced response; n=2 to 3 animals/genotype with 7 to 11 cells in each group. Statistical significance was determined by 1-way ANOVA followed by Newman–Keuls multiple comparison test. *P<0.05 vs WT; #P<0.05 vs respective basal.

Ca²⁺ Leak and Ca²⁺ Handling Proteins
Because isoproterenol-induced arrhythmias are to a large extent caused by an increased diastolic Ca²⁺ leak from the SR via the ryanodine receptor (RYR2), analysis of Ca²⁺ sparks was conducted to estimate Ca²⁺ leakage. Phosphodiesterase 2-TG
animals revealed a trend to a lower number of sparks under basal conditions and a complete abrogation of the increase in spark frequency after application of isoproterenol, indicating the likely underlying cause of the reduced arrhythmia burden of phosphodiesterase 2-TG mice after isoproterenol injection (Figure 7A and 7B). This was further confirmed by assessing the SR Ca\(^{2+}\) leak as the difference between the Fura-2 ratio with and without RYR2 blocker tetracaine (1 mmol/L), using a 0Na+/0Ca\(^{2+}\) solution to prevent Ca\(^{2+}\) extrusion by the Na+/Ca\(^{2+}\) exchanger (Online Figure IX).

To explore the molecular mechanisms of a reduced Ca\(^{2+}\) leak, we performed immunoblot analysis of key Ca\(^{2+}\) handling proteins (Figure 7C through 7G). Consistent with the preserved SR Ca\(^{2+}\) load in phosphodiesterase 2-TG mice (Figure 5D), we did not find any differences between WT and phosphodiesterase 2-TG mice regarding the expression of SERCA2a, phospholamban, or RYR2. Phospholamban phosphorylation was significantly reduced at S16 and milder also at T17. Most strikingly, we found a reduction of RYR2 receptor phosphorylation at the described Ca\(^{2+}\)/calmodulin-dependent kinase II phosphorylation site, S2814, which has been linked to diastolic Ca\(^{2+}\) leakage, but not at the putative PKA site S2808.

**Cardiac Function and Arrhythmia After MI**

To analyze the protective potential of phosphodiesterase 2 under chronic stress conditions, mice were subjected to ligation of the left anterior descending artery to induce MI. Although infarct size was similar in both groups (Figure 8C), phosphodiesterase 2-TG were markedly protected against ventricular failure with an ejection fraction of 47±5\% compared with 31±4\% in WT (Figure 8A and 8B) at 14 days after MI. The overall VT incidence was only slightly and not significantly lower in phosphodiesterase 2-TG mice than that in WT: 62.5\% of the WT and 53.3\% of phosphodiesterase 2-TG developed VTs in the first 40 hours after MI (Figure 8E and 8H). However, only 30\% of WT with VTs survived the first 7 days, whereas none of the phosphodiesterase 2-TG mice with VTs had an early death (Figure 8F). Moreover, all early WT deaths were preceded by VTs, whereas phosphodiesterase 2-TG did not show this correlation (Figure 8G). Overall, >86\% of phosphodiesterase 2-TG survived the first 7 days post MI, whereas only 56\% of WT animals endured >7 days (Figure 8D; P=0.06). No further deaths occurred in either group between day 8 and day 14 (end point of intervention).

**Discussion**

Myocardial phosphodiesterase 2 is upregulated in human and in experimental HF, but its physiological and pathologi- cal role in the heart remained unknown. Here, we show that heart rate regulation is the predominant physiological role of phosphodiesterase 2. Specific inhibition of phosphodiesterase 2 in dogs and mice led to an exclusive increase in heart rate, whereas overexpression of phosphodiesterase 2 resulted in its decrease. Under chronic β-AR activation, however, phosphodiesterase 2 contributes to myocardial β-AR desensitization, protecting the heart from excessive sympathetic stress. Moreover, under acute β-adrenergic stress, higher phosphodiesterase 2 abundance effectively protects against ventricular arrhythmia without compromising contractile
Role of Phosphodiesterase 2 in Heart Rate Regulation

The modern concept of heartbeat initiation is based on the mutual interplay between ion channels of the cell membrane (membrane clock) and cellular Ca\(^{2+}\) cycling (Ca\(^{2+}\) clock).\(^{31,44}\) The most prominent targets of sympathetic heart rate modulation are the funny current (I\(_{\text{f}}\)), mediated by cAMP-dependent regulation of HCN channels and PKA-dependent phosphorylation of L-type Ca\(^{2+}\) channels and of SR Ca\(^{2+}\) cycling proteins.\(^{45}\) To date, phosphodiesterase 2 has been shown to contribute to myocardial Ca\(^{2+}\) cycling by modulating I\(_{\text{Ca,L}}\), not only in ventricular but also in atrial and sinoatrial nodal cells.\(^{33,24,46}\) These findings are consistent with the attenuated β-AR responsiveness regarding I\(_{\text{Ca,L}}\) and SR Ca\(^{2+}\) release observed in phosphodiesterase 2-TG–derived ventricular myocytes. Moreover, the close interplay between I\(_{\text{Ca,L}}\), RYR2-mediated Ca\(^{2+}\) release, and SERCA/phospholamban-dependent filling of the SR suggests that phosphodiesterase 2 is involved in the regulation of the Ca\(^{2+}\) clock.\(^{44}\) However, although in vivo contraction force was unaffected, phosphodiesterase 2-TG exhibited lower basal and maximal heart rates but retained β-AR–induced control of pacemaker activity. This particular chronotropic phenotype has remarkable parallels with a cardiac cAMP-binding deficient HCN4 mutation analyzed in mice and humans.\(^{38,47}\) Notably, although HCN-blockade with ivabradine had no effect in mice expressing the mutated channel,\(^{47}\) phosphodiesterase 2-TG mice were still sensitive
to ivabradine treatment, indicating a remaining contribution of \( I_f \) to basal heart rate regulation. This study shows for the first time that phosphodiesterase 2 is a major player in heart rate regulation in vivo, most likely by affecting both membrane and \( Ca^{2+} \) clock.

Role of Phosphodiesterase 2 in Propensity to Arrhythmia and in Contractility

There is substantial evidence that generation of delayed afterdepolarizations because of increased diastolic \( Ca^{2+} \) leak from the SR via RYR2 and the subsequent depolarizing activity of the

Figure 8. Phosphodiesterase 2 (PDE2)-transgenic (TG) are protected from early death, sustained arrhythmias, and decline of heart function after myocardial infarct (MI). A, Ejection fraction calculated from echocardiographic analysis before MI (baseline) and at day 14 post MI. B, Representative M-Mode trace from infarcted area taken 14 d post MI. C, Left: Representative sirius red staining of heart sections prepared from surviving animals at day 14; right: quantification of infarct size given as % of total tissue area. D, % of surviving animals. Study was terminated 14 d post MI. E–G, Analysis of ventricular tachycardia (VT) during the first 40 h post MI and association with early death events. H, Representative ECG traces showing a 17 s lasting VT in WT and 4 s in transgenic (TG) animals. Statistical significance was determined by 1-way ANOVA followed by Newman–Keuls multiple comparison test (A), log-rank test (B), and Fischer exact test (F and G). *P<0.05 vs WT, #P<0.05 vs respective baseline.
Na+/Ca2+ exchanger is the main underlying mechanism for triggered arrhythmias. A central role in this dysfunction of Ca2+ cycling has been attributed to the phosphorylation of RYR2 at the calmodulin-dependent kinase II site S2814 and the associated facilitation of diastolic Ca2+ release.41-43,45 Our conclusion that phosphodiesterase 2-TG mice are less susceptible to arrhythmia provocation induced by acute β-AR stimulation because of a significantly lower Ca2+-spark frequency and lower RYR2-S2814 phosphorylation fits well to this model. At the cardiomyocyte level, efficacy of β-AR–induced increase in sarcomere shortening was significantly attenuated, as were efficacy and potency of β-AR–induced stimulation of Ca2+ transients. Notably, at baseline and at low isoproterenol concentrations, contractile parameters were normal (Online Figure VIII). However, the in vitro findings on force development did not entirely recapitulate the actual phenotype of phosphodiesterase 2-TG mice. Our in vivo data clearly demonstrate that phosphodiesterase 2 overexpression was associated with a normal contractile reserve and rather improved contraction force. This was even maintained when heart rate was reduced to a similar level induced by acute β-blockade (Figure 2D through 2F), indicating that the lower basal heart rate per se, for example, via augmented filling in diastole, cannot completely explain the hypercontractile phenotype. Despite lower phospholamban phosphorylation, SR load was not affected in phosphodiesterase 2-TG. Longer diastolic intervals and the reduced Ca2+ leak may, therefore, be sufficient for maintaining adequate SR Ca2+ filling and preservation of cardiac function in vivo, even in the presence of reduced SERCA activity.48 In summary, phosphodiesterase 2 overexpression offers a potential dual protection by limiting heart rate without affecting chronotropic adaptation and by attenuating ventricular SR Ca2+ release with the benefit of lower arrhythmia susceptibility. The effect of phosphodiesterase 2 overexpression may, therefore, involve some features that β-blocker therapy is well known for but without depression of contractile performance.

Phosphodiesterase 2 in Cardiac Remodeling

A recent publication proposed that in the context of cardiac remodeling processes, chronic inhibition of phosphodiesterase 2 leads to a reduction of pathological hypertrophic growth.50 Although this contradicts our earlier finding that adenoviral overexpression of phosphodiesterase 2 antagonizes β-AR–induced cellular hypertrophy,29,51 we observed a small but not significant increase in cardiac size in phosphodiesterase 2-TG mice as compared with WT (Figures 2G and 3C). Therefore, we cannot completely rule out a minor increase in heart size because of phosphodiesterase 2 overexpression. Consistently, heart weight was also ≈10% higher in phosphodiesterase 2-TG mice after MI compared with WT (Online Figure X). However, preservation of cardiac function and size up to an advanced age (Figure 3) strongly argues against pathological hypertrophy. Moreover, we offer proof that high abundance of phosphodiesterase 2 significantly protects against acute and chronic β-AR stress and maintains contractile function after MI.

Potential Limitations

A general limitation of transgenic overexpression is potential spill over within subcellular compartments, where the protein of interest may not be physiologically located. This is even more critical when examining phosphodiesterases, which control highly compartmentalized cAMP pools, and redistribution phenomena under pathological conditions have been reported.52,53 Despite designing our experiments after general recommendations for state-of-the-art phenotyping of transgenic mice,54,55 we are not able to fully exclude artificial compartmentation effects. However, the specificity of the phenotype and its striking similarities to in vivo studies of endogenous phosphodiesterase 2 from mice and larger animals offers a valid approach for analyzing the pathophysiological role of phosphodiesterase 2 in heart function. A second limitation is that the role played by each phosphodiesterase isoform varies among mammalian species,54,55 and accordingly, our results may not recapitulate the situation in humans in all details.

Clinical Perspective: Phosphodiesterase 2 as a Downstream Target of cGMP Pools

The current therapeutic strategies for HF and prevention of sudden cardiac death are only moderately efficient. Despite all efforts, a limited understanding of the pathophysiological mechanisms underlying HF and arrhythmias has hindered the development of more effective, rational therapeutic approaches. Recently, the publication of the PARADIGM-HF trial (Prospective Comparison of ARNI with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure), which demonstrated the successful introduction of the compound LCZ696 (a combination of a standard angiotensin-II receptor-1 blocker and an inhibitor of the natriuretic peptide–degrading enzyme neprilysin), has once again shifted the enhancement of cGMP signaling into the focus of HF therapy. One remarkable result of the study was a significant protection from sudden cardiac death.56,57 The importance of the natriuretic peptide–signaling pathway was further emphasized in a study by Lee et al16 showing that the inhibition of cGMP-degrading phosphodiesterase 9 protects against HF progression by specifically targeting the ANP/BNP (atrial natriuretic peptide/B-type natriuretic peptide)-coupled cGMP pool.58 Phosphodiesterase 2 is a central component of the cGMP/cAMP crosstalk and, as our study demonstrates, effectively protects against ventricular arrhythmia during excessive sympathetic stress and improves ventricular function after severe cardiac insult. It may, therefore, constitute an up to now unconsidered link between ANP/BNP-coupled cGMP enhancement and protection against toxic sympathetic effects by acting as a cGMP-controlled intracellular sympathetic blockade. Thus, phosphodiesterase 2 is worth being considered a key element in recent encouraging therapeutic approaches, and accordingly, its direct activation may offer an alternative strategy in a promising new field of HF therapy.

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Disclosures
None.

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Supplemental Material

Phosphodiesterase 2 Protects against Catecholamine-induced Arrhythmia and Preserves Contractile Function after Myocardial Infarction

Running title: Vettel et al., PDE2 in arrhythmia and contractile function

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Detailed Methods

All experiments were carried out according to the European Community guiding principles in the care and use of animals (2010/63/UE, 22 September 2010), the local Ethics Committee (CREEA Île-de-France Sud) guidelines and the French decree n° 2013-118, 1st February 2013 on the protection of animals used for scientific purposes (JORF n°0032, 7 February 2013 p2199, text n° 24). Authorizations were obtained from the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Germany) and Ministère Français de l'Agriculture, de l'Agroalimentaire et de la Forêt (agreement N°B 92-019-01).

Application of BAY 60-7550 for cardiovascular analysis, canine animal model

Evaluation of hemodynamic parameters was performed in beagle dogs according to GLP requirements using a dog model described earlier\(^1\). Both male and female animals were used at an age of 1 to 5.5 years and 9.5 to 16 kg body weight. Briefly, 12 Beagle dogs were subjected to general neuroleptic anesthesia (droperidol + fentanyl) and mechanical ventilation with nitrous oxide/oxygen (1:3). Administration formulations of BAY 60-7550 (BAY) were prepared in ethanol/polyethylene glycol 400 (1:9 v/v) and administered intraduodenally (i.d.) with an administration volume of 1-2 ml/kg in a dose range of 3, 10, and 30 mg/kg (n=3 dogs per group). Three control animals received the vehicle only. Dogs were instrumented with a Millar tip catheter placed into the abdominal aorta for measurement of systemic arterial blood pressure. A second Millar catheter equipped with a pressure and a velocity sensor was introduced into the heart via the left carotid artery. The pressure sensor was located within the left ventricle, the velocity sensor located in the ascending aorta to allow the measurement of stroke volume, left ventricular pressure (LVP) and the determination of left ventricular pressure rise (LV dP/dt), a surrogate for heart contractility. Heart rate was determined by ECG. Cardiac output (CO) and total peripheral resistance were calculated from stroke volume, heart rate and mean arterial blood pressure. At predefined time points at baseline and up to 240 min after administration, cardiovascular parameters were collected, stored and evaluated using P3 Plus Ponemah software (DSI).

Chronic isoproterenol administration

Isoproterenol (ISO, Sigma-Aldrich) was delivered to mice by subcutaneously implanted osmotic minipumps (Alzet, model 2002) that released ISO solved in 0.9% NaCl at a dose of 30 μg/g/d\(^2\). Anesthesia was performed with isoflurane (1.5% v/v). After 7 days, cardiac function was monitored by echocardiography. The mice used for this study were 2 month old littermates with a FVB/N background. Groups were age and sex matched.

Echocardiography

Animals were kept under light temperature and ECG-controlled anesthesia (isoflurane, 1.5% v/v or pentobarbital 35 mg/kg body weight (i.p.)) during the whole procedure. Echocardiography images (Vevo 770® System or 2100® System (MI), Visual Sonics Inc.) were obtained in a parasternal long and a short axis view at midpapillary and apical (representative M-mode pictures, MI) muscle level at a frame rate of 60 Hz. Long axis images were used to measure left ventricle length (L) during end-diastole (d) and end-systole (s). The thickness of the anterior (AWTh) and posterior wall (PWTh), the left ventricular diameter (LVD), the epicardial (EpiA) and endocardial (EndoA) area of the left ventricular cavity were obtained in the short axis or long axis (MI) view during d and s stages. Parameters were calculated as follows: \%Fractional area shortening (%FAS) = (EndoAd – EndAs)/EndoAd x 100; systolic volume (SV) = 5/6 x (EndoAd x Ld – EndoAs x Ls); cardiac output CO=SV x HR/1000; left ventricular weight (LVW) = 1.05 x 5/6 x [EpiAs x (Ls + (AWThs + PWThs)/2)] – EndoAs x Ls, where 1.05 is the specific gravity of muscle. \%Ejection fraction = 100\(^*\) \(((7.0 / (2.4 + average diastolic diameter)+average diastolic diameter\(^3\)) / ((7.0 / (2.4 + average diastolic diameter) + average systolic diameter\(^3\)) (average diastolic diameter\(^3\))\(^3\)). In MI, measurements were performed before and two weeks after left anterior descending coronary artery ligation.
Longevity study
Animals were kept under standard housing conditions until either natural death or any severe illness occurred (e.g. tumor growth, colic, age related decay etc.). Animals, which fell sick during the study were euthanized according to animal care guidelines to avoid unnecessary pain and counted as a naturally death event. The study was terminated after 38 months with 3 still living individuals. Groups were age and sex matched.

Implantation of ECG transmitters
Mice were anaesthetized with isoflurane (2% v/v) via mask ventilation and placed on a warming plate (37°C). After the skin of the anterior thoracic region was depilated and disinfected, a 2 cm long median incision of the thoracic skin was made. The underlying tissue was prepared in order to create subcutaneous space for the ECG-transmitter (Data Sciences International, ETA-F10) and the electrodes. Afterwards, the ECG-transmitter was placed subcutaneously to the back of the mouse, the negative electrode was fixed to the right pectoralis fascia and the positive electrode was fixed 1 cm left to the xiphoid. The wound was closed using resorbable sutures. Alternatively, the transmitters were implanted into the peritoneal cavity. Buprenorphine 0.05 mg/kg s.c. once before starting the surgical procedure and metamizol 300 mg/kg p.o. from 2 days before to 7 days after the surgical procedure were used for intra- and postoperative analgesia. Recordings were started after a recovery time of at least two weeks post subcutaneous implantation of the telemetric transmitter). Recording and analysis parameters were set according to the manufacturer’s instructions using P3 Plus software (DSI) or LabChart software (Chart 5.4, AD Instruments) and to conventional arrhythmia/frequency analysis guidelines3-5. Heart rate, activity and RR-intervals are given as either an average of 1 min or of 5 s intervals. Details are specified in the respective figure legends.

Isolation of adult mouse cardiomyocytes
Ventricular myocytes were obtained from 10 to 14 week old male mice. Animals were anesthetized by intraperitoneal injection of pentothal (150 mg/kg), and the heart was quickly removed and placed into cold Ca\(^{2+}\)-free Tyrode’s solution containing (in mmol/L): NaCl 113, KCl 4.7, MgSO\(_4\) 4, KH\(_2\)PO\(_4\) 0.6, NaH\(_2\)PO\(_4\) 0.6, BDM 10, NaHCO\(_3\) 1.6, HEPES 10, Taurine 30, D-glucose 20, adjusted to pH 7.4. The ascending aorta was cannulated and the heart was perfused with oxygenated Ca\(^{2+}\)-free Tyrode’s solution at 37°C during 4 min. For enzymatic dissociation, the heart was perfused with Ca\(^{2+}\)-free Tyrode’s solution containing liberase TM research grade (Roche Diagnostics) for 10 min at 37°C. Then the heart was removed and placed into a dish containing Tyrode’s solution supplemented with 0.2 mmol/L CaCl\(_2\) and 5 mg/ml BSA (Sigma-Aldrich). The ventricles were separated from the atria, cut into small pieces, and triturated with a pipette to disperse the myocytes. Ventricular myocytes were filtered on gauze and allowed to sediment by gravity for 10 min. The supernatant was removed and cells were suspended in Tyrode’s solution supplemented with 0.5 mmol/L CaCl\(_2\) and 5 mg/ml BSA. The procedure was repeated once and cells were suspended in Tyrode’s solution with 1 mmol/L CaCl\(_2\). Freshly isolated ventricular myocytes were plated in 35 mm culture dishes coated with laminin (10 µg/ml) and stored at room temperature until use6.

cAMP measurements by FRET
Adult mouse ventricular myocytes isolated from WT or PDE2-TG mice were infected with an adenovirus encoding the Epac-SH187 cAMP FRET probe for 24 h (kindly provided by Dr. Kees Jalink, Cancer Institute, Amsterdam, The Netherlands)7. Thereafter, the cells were washed once and maintained in a physiological buffer containing (in mmol/L): NaCl 144, KCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 1.8, and HEPES 20, pH 7.4 at room temperature. Images were captured every 5 s using the ×40 oil immersion objective of an inverted microscope (Nikon) connected to a Cool SNAP HQ2 camera (Photometrics) controlled by the Metafluor software (Molecular Devices). Cyan Fluorescent Protein (CFP) was excited for 300 ms by a Xenon lamp (Nikon) using a 440/20BP filter and a 455LP dichroic mirror. Dual-emission imaging of CFP and Yellow Fluorescent Protein (YFP) was performed using a Dual-View emission splitter.
equipped with a 510 LP dichroic mirror and BP filters 480/30 and 535/25 nm, respectively. The YFP/CFP emission ratio upon 436 nm excitation (filters YFP 535 ± 15 nm, CFP 480 ± 20 nm) was measured. After each measurement, emission values were corrected for bleedthrough of CFP into the YFP channel. The imaging data was analyzed with Excel. All experiments were performed at room temperature.

**I_{Ca,L} measurements**

The whole-cell configuration of the patch-clamp technique was used to record I_{Ca,L}. Pipette resistance was between 1–2 MΩ when filled with internal solution containing (in mmol/L): CsCl 118, EGTA 5, MgCl₂ 4, sodium phosphocreatine 5, Na₂ATP 3.1, Na₃GTP 0.42, CaCl₂ 0.062 (pCa 8.5), HEPES 10, adjusted to pH 7.3 with CsOH. Extracellular Cs⁺-Ringer solution contained (in mmol/L): CaCl₂ 1.8, MgCl₂ 1.8, NaCl 107.1, CsCl 20, NaHCO₃ 4, NaH₂PO₄ 0.8, D-glucose 5, sodium pyruvate 5, HEPES 10, adjusted to pH 7.4 with NaOH. For I_{Ca,L} measurement, the cells were depolarized every 8 s from -50 to 0 mV for 400 ms and the maximal amplitude of whole-cell I_{Ca,L} was measured as previously described⁸. The use of -50 mV as holding potential allowed the inactivation of voltage dependent sodium currents. K⁺ currents were blocked by replacing all K⁺ ions with external and internal Cs⁺. Currents were not compensated for capacitance and leak currents. All experiments were performed at room temperature.

**Measurements of Ca²⁺ transients, sarcomere shortening, SR Ca²⁺ leak and load**

All experiments were performed at room temperature within 6 h after cell isolation. Isolated mouse ventricular cardiomyocytes were loaded with 3 µmol/L Fura-2 AM (Invitrogen) for 15 min in Ringer’s solution containing (in mmol/L): KCl 5.4; NaCl 121.6; Na-pyruvate 5; NaHCO₃ 4.013; NaH₂PO₄ 0.8; CaCl₂ 1.0; MgCl₂ 1.8; glucose 5 and HEPES 10 (pH 7.4 with NaOH). Sarcomere shortening and Fura-2 ratio (measured at 512 nm upon excitation at 340 and 380 nm) were simultaneously recorded in Ringer’s solution, using a double excitation spectrofluorimeter coupled with a video detection system (IonOptix, Milton, MA, USA). Myocytes were electrically stimulated with biphasic field pulses (5 V, 4 ms) at a frequency of 0.5 Hz as previously described⁹. Because arrhythmias depend on the initial quality of cells, cardiomyocytes exhibiting spontaneous Ca²⁺ waves (sCaW) when perfused with control Ringer solution were discarded.

SR Ca²⁺ leak and load were measured according to a dedicated protocol¹⁰. Fura-2 loaded ventricular myocytes were paced by field stimulation at 0.5 Hz in normal Ringer’s for few minutes until cellular Ca²⁺ transients reached a steady state. Directly after the last pulse, normal Ringer’s was substituted for 30 s by a 0Na⁺/0Ca²⁺ Ringer’s in which Na⁺ was replaced by Li⁺ and supplemented with 10 mmol/L EGTA. This condition allowed measuring intracellular Ca²⁺ levels in a closed system without trans-sarcolemmal Ca²⁺ fluxes. Then, the cell was switched back to normal Ringer’s and paced at 0.5 Hz until Ca²⁺ transient amplitude and sarcomere shortening reached steady-state. Again, following the last pulse, cells were perfused for 30 s with a 0Na⁺/0Ca²⁺ solution including 1 mmol/L of the RyR2 inhibitor tetracaine. As a consequence, SR Ca²⁺ leak into the cytoplasm was prevented. SR Ca²⁺ leak was estimated as the difference between the Fura-2 ratio recorded at the end of the 0Na⁺/0Ca²⁺ Ringer’s perfusion with and without tetracaine. SR Ca²⁺ leak was washed out for at least 60 seconds and 10 mmol/L caffeine was applied to evaluate the total SR Ca²⁺ content.

Ca²⁺ transient amplitude was measured by dividing the twitch amplitude (difference between the end-diastolic and the peak systolic ratios) by the end-diastolic ratio, thus corresponding to the percentage of variation in the Fura-2 ratio. Similarly, sarcomere shortening was assessed by its percentage of variation, which is obtained by dividing the twitch amplitude (difference between the end-diastolic and the peak systolic sarcomere length) by the end-diastolic sarcomere length. Relaxation was assessed by measuring the time-to-50% relaxation from the time to peak shortening, and the Ca²⁺ transient decay was evaluated by measuring the time-to-50% decay of the Fura-2 ratio from the time to peak ratio. SR Ca²⁺ leak was measured by subtracting the ratio of fluorescence recorded in steady-state in
0Na*/0Ca²⁺ Ringer’s with tetracaine from the ratio recorded in steady-state in 0Na*/0Ca²⁺ Ringer’s without tetracaine. SR Ca²⁺ load was estimated by dividing the amplitude of the caffeine-induced twitch (difference between the peak ratio obtained with caffeine and the diastolic ratio measured before tetracaine treatment) by the diastolic ratio. Fractional release was calculated by dividing the Ca²⁺ transient amplitude by the caffeine-induced twitch amplitude, thus corresponds to the fraction of Ca²⁺ released from the SR during a twitch. All parameters were calculated offline using IonWizard 6 (IonOptix).

**Ca²⁺ spark analysis**

Mice were sacrificed under isoflurane anesthesia (5% v/v) by cervical dislocation. 100 I.U. heparin was administered by intraperitoneal injection prior to isolation, to ensure sufficient perfusion of myocardium. Explanted hearts were retrogradely perfused on a Langendorff system, first with a Ca²⁺ free solution containing (in mmol/L) NaCl 113, KCl 4.7, KH₂PO₄ 0.6, Na₂HPO₄ x2H₂O 0.6, MgSO₄ x7H₂O 1.2, NaHCO₃ 12, KHCO₃ 10, HEPES 10, taurine 30, BDM 10, glucose 5.5, phenol-red 0.032 (37°C, pH 7.4), followed by the addition of 7.5 mg/ml liberase 1 (Roche diagnostics) and trypsin 0.6% (Life Technologies) as well as 0.125 mmol/L CaCl₂. Upon becoming flaccid, ventricular and atrial myocardium were separated. Ventricular myocardium was cut into small pieces and dispersed in solution. Ca²⁺ concentration was increased in steps every 7 min until desired concentration was reached. Cells were plated on laminin-coated recording chambers and left to settle for 20 min.

Isolated mouse ventricular cardiac myocytes were incubated for 15 min at room temperature with a Fluo-3 AM loading buffer (10 μmol/L, Molecular Probes). Experimental solution contained (in mmol/L): KCl 4, NaCl 140, MgCl₂ 1, HEPES 5, glucose 10, CaCl₂ 2 (pH 7.4, NaOH, room temperature) plus isoproterenol (ISO) 100 nmol/L for the ISO experiments. Myocytes were superfused with experimental solution for 5-10 min before experiments commenced and during experiments to remove excess indicator and to allow time for complete deesterification of Fluo-3 AM. Ca²⁺ spark measurements were carried out on a laser scanning confocal microscope (LSM 5 Pascal, Zeiss) with a 40x oil-immersion objective. Fluo-3 was excited by an argon ion laser (488 nm). Emitted fluorescence was collected through a 505 nm long-pass emission filter. Fluorescence images were recorded in the line-scan mode (width of scan line: 38.4 μm, 512 pixels per line, pixel time: 0.64 μs, number of unidirectional line scans: 10,000, measurement period: 7.68 s). Confocal line scans were performed at rest after a brief period of field stimulation to load the SR (10 pulses, 1 Hz, 20 V). Ca²⁺ sparks were analyzed in SparkMaster for ImageJ. Mean spark frequency (CaSpF) was normalized to cell width and scan rate (100 μm⁻¹ s⁻¹).

**Immunoblot analysis**

Protein samples were prepared from pulverized ventricular myocardium and lyzed in buffer containing 30 mmol/L Tris/HCl (pH 8.8), 5 mmol/L EDTA, 30 mmol/L NaF, 3% SDS, and 10% glycerol. Samples were separated in denaturing acrylamide gels and subsequently transferred onto nitrocellulose or PVDF membranes. After blocking the membranes with Roti®-block (Carl Roth) for 1 h, the incubation with anti-PDE2 (1:1,000, FabGennix), anticalsequestrin (1:1,000, ThermoScientific), and anti-α-tubulin (1:2,000, Sigma-Aldrich) was carried out over night at 4°C. After incubation with appropriate secondary antibodies for 1 h, proteins were visualized by enhanced chemoluminescence and quantified with Quantity One software (Biorad).

**Measurement of infarct size**

Hearts were fixed in 4% (m/V) paraformaldehyde and embedded in paraffin. Hearts were sliced transversely (2 μm). Sections of the midpapillary region were stained with Sirius Red as described previously. For the determination of the infarct size, the epicardial and endocardial infarct length and circumference was measured. Infarct size was calculated as follows: [(epicardial infarct length/ epicardial circumference) + (endocardial infarct length/ endocardial circumference)/2] * 100.
Online Figures

Online Figure I. ISO infusion induces left ventricular hypertrophy. Relative left ventricular weight (LVW) determined by echocardiography to body weight (BW) ratio in mice subjected to chronic ISO infusions (30 mg/kg/d for 7d) or NaCl (0.9%) as control; n=7-9. Statistical significance was determined by Student’s t-test. *p<0.05 vs. NaCl.
Online Figure II. Effect of PDE2-specific inhibitor BAY 60-7550 on hemodynamic parameters in beagle dogs. Effect of PDE2 inhibition in dogs exposed to the indicated doses of BAY 60-7550 (i.d.). Animals were anaesthetized, equipped with catheters (abdominal aorta, left ventricle and ascending aorta) and monitored by echocardiography over a period of 240 min post application. (A) Maximal increase in heart rate (HR) given as % over respective basal HR. (B) Table with assessed hemodynamic parameters at the time point of maximal increase in heart rate (HR): systolic and diastolic blood pressure (BPs, BPd), left ventricular pressure (LVP), cardiac output (CO), stroke volume (SV). Average of n=3 for each group. Statistical significance was determined by one-way ANOVA. *p<0.05 for linear trend.
Online Figure III. Characterization of the lower expressing transgenic mouse line TG-4808. Echocardiographic determination of fractional area shortening (FAS, A), cardiac output (CO, B), and heart rate (HR, C) in anaesthetized 2 month old mice. Animals were treated with 10 mg/kg dobutamine (DOBU, i.p.) 2 min prior to measurements when indicated. (D) Left ventricular weight (LVW) calculated from the echocardiographic data and normalized to tibia length; n=12-14 for each group. (E, F) Lysates prepared from ventricular myocardium were analyzed by immunoblot with the indicated specific antibodies. PDE2 expression was normalized to tubulin and given relative to WT. (E) Immunoblots and (F) quantification; n=2 for each group Statistical significance was determined by one-way ANOVA followed by Newman-Keuls multiple comparison Test (A, C) and by Student’s t-test (B, D). #p<0.05 vs. respective basal.
Online Figure IV. Characterization of the high expresser transgenic mouse line TG-4811. Echocardiographic determination of fractional area shortening (FAS, A), cardiac output (CO, B), and heart rate (HR, C) in anaesthetized 2 month old mice. Animals were additionally treated with 10 mg/kg dobutamine (DOBU, i.p.) 2 min prior to measurements when indicated. (D) Left ventricular weight (LVW) calculated from the echocardiographic data and normalized to body weight (BW); n=6-8 for each group. (E, F) Lysates prepared from ventricular myocardium were analyzed by immunoblot with the indicated specific antibodies. PDE2 expression was normalized to tubulin and given relative to WT. (E) Immunoblots and (F) quantification; n=2 for each group. Statistical significance was determined by one-way ANOVA followed by Newman-Keuls multiple comparison test (A, C) and by Student's t-test (B, D). *p<0.05 vs. WT, #p<0.05 vs. respective basal.
Online Figure V. Metoprolol reduced heart rate to a similar extent in WT and PDE2-TG, while basal hypercontractility of PDE2-TG was independent of β-AR activity. Echocardiographic determination of heart rate (HR, A) and fractional area shortening (FAS, C) in anaesthetized mice in the presence of increasing metoprolol doses (1, 3, 10, 30, 100 mg/kg, i.p.). (B, D) Normalization of A and C to compare potency.
Online Figure VI. ECG-Telemetry: Activity pattern and Poincaré plots of RR intervals documented over a period of 24 h. Application of HCN-Blocker Ivabradine (IVA).

Animals (n=4 per genotype) were monitored by ECG-telemetry for a period of 72 h to calculate average changes in activity of a 24 h cycle. Activity was tracked as an average of 1 min intervals. (A) Average circadian changes in activity. (B) Average activity during day and night periods. Statistical significance was determined by one-way ANOVA followed by Newman-Keuls multiple comparison test. *p<0.05 vs. respective basal. (C, D) Representative Poincaré plot of a WT and TG animal regarding the distribution of daily RR intervals. Sinus arrest and AV block were excluded. (E) Average traces of IVA-application 45 min prior and 2 h after ivabradine (IVA) injection (5 mg/kg, i.p.). Frequencies were tracked as average of 5 s intervals; n=5.
Online Figure VII. PDE2 overexpression attenuates β-AR stimulation of Ca²⁺ transients and sarcomere shortening in cardiomyocytes. Adult ventricular myocytes isolated from WT or PDE2-TG mice were loaded with Fura-2 and stimulated at a frequency of 0.5 Hz in the absence or presence of increasing concentrations of ISO. Sarcomere length and Fura-2 ratio were recorded using an IonOptix System. Concentration-response relationship was extrapolated for WT cardiomyocytes under the assumption that 100 nmol/L ISO is sufficient for maximal responsiveness. (A) Average amplitudes of sarcomere shortening. (B) Normalization of (A) to compare logEC₅₀ values with lowest values set to 0% and highest values set to 100%. (C) Average amplitudes of Ca²⁺ transients. (D) Normalization of (C) to compare logEC₅₀ values with lowest values equal 0% and highest values equal 100%. (E) Average amplitudes of half-time relaxation of sarcomere shortening (t₁/₂ off). (F) Average
amplitudes of half-time relaxation of Ca\(^{2+}\) transients (t\(_{1/2}\) off). n=3-6 animals/genotype with 6-20 cells in each group. Data sets were subjected to comparison of fit (extra sum-of-squares F test) regarding top values (efficacy) or logEC\(_{50}\) (potency). *p<0.05 vs. WT.
Online Figure VIII. Arrhythmia study in healthy mice. Heart rate was monitored after double ISO injection (2 mg/kg, i.p.; time interval between injections 30 min) and analyzed for arrhythmic events such as ventricular extra systoles, bigeminy, salvos and VTs over a period of 90 min; n=7. (A) Average ISO-induced ventricular extra systoles (VES including salvos and ventricular tachycardia) per minute. Regular occurrence of VES 15 min after the second injection of ISO in all animals. (B) Occurrence of bigeminy (BG) and (C) salvos in addition to main Fig. 4. Statistical significance was determined by Fisher’s exact test to compare occurrence of events.
Online Figure IX. SR Ca\textsuperscript{2+} leak (A) Representative traces of Ca\textsuperscript{2+} transients, sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} leak and load measured in Fura-2 loaded adult mouse ventricular myocytes from WT (left) or PDE2-TG mouse (right) paced at 1 Hz, upon ISO (100 nmol/L) application. Tetracaine (1 mmol/L) was used to estimate SR Ca\textsuperscript{2+} leak, caffeine (10 mmol/L) to measure SR Ca\textsuperscript{2+} load. (B) Mean amplitude of the SR Ca\textsuperscript{2+} leak recorded in control or ISO. n=3 animals/genotype with 7-10 cells in each group. Statistical significance was determined by one-way ANOVA followed by Newman-Keuls multiple comparison test. *p<0.05 vs. WT, #p<0.05 vs. respective basal.
Online Figure X. Echocardiographic parameters and heat weights after MI.
Echocardiographic analysis 14 d post MI of (A) heart rate, (B) stroke volume, (C) left
ventricular inner end-diastolic diameter and (D) left ventricular posterior wall end-diastolic diameter. Biometric data included determination of (E) heart weight (HW) to tibia length ratios, (F) lung weight (LW) to tibia length ratios, (G) body weight (BW) and (H) tibia length. Statistical significance was determined by Student's t-test. *p<0.05 vs. WT.
Supplemental References


