

Transgenic Mice for Real-Time Visualization of cGMP in Intact Adult Cardiomyocytes

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Rationale: 3',5'-Cyclic guanosine monophosphate (cGMP) is an important second messenger that regulates cardiac contractility and protects the heart from hypertrophy. However, because of the lack of real-time imaging techniques, specific subcellular mechanisms and spatiotemporal dynamics of cGMP in adult cardiomyocytes are not well understood.

Objective: Our aim was to generate and characterize a novel cGMP sensor model to measure cGMP with nanomolar sensitivity in adult cardiomyocytes.

Methods and Results: We generated transgenic mice with cardiomyocyte-specific expression of the highly sensitive cytosolic Förster resonance energy transfer–based cGMP biosensor red cGES-DE5 and performed the first Förster resonance energy transfer measurements of cGMP in intact adult mouse ventricular myocytes. We found very low (≈ 10 nmol/L) basal cytosolic cGMP levels, which can be markedly increased by natriuretic peptides (C-type natriuretic peptide $\gg$ atrial natriuretic peptide) and, to a much smaller extent, by the direct stimulation of soluble guanylyl cyclase. Constitutive activity of this cyclase contributes to basal cGMP production, which is balanced by the activity of clinically established phosphodiesterase (PDE) families. The PDE3 inhibitor, cilostamide, showed especially strong cGMP responses. In a mild model of cardiac hypertrophy after transverse aortic constriction, PDE3 effects were not affected, whereas the contribution of PDE5 was increased. In addition, after natriuretic peptide stimulation, PDE3 was also involved in cGMP/cAMP crosstalk.

Conclusions: The new sensor model allows visualization of real-time cGMP dynamics and pharmacology in intact adult cardiomyocytes. Förster resonance energy transfer imaging suggests the importance of well-established and potentially novel PDE-dependent mechanisms that regulate cGMP under physiological and pathophysiological conditions. (*Circ Res.* 2014;114:1235-1245.)

Key Words: biosensing techniques ■ fluorescence resonance energy transfer ■ hypertrophy ■ mice, transgenic ■ myocytes, cardiac ■ phosphodiesterase inhibitors

3',5'-Cyclic guanosine monophosphate (cGMP) is one of the ubiquitous second messengers, which is critically involved in the regulation of cardiac contractility and pathological hypertrophy.^{1,2} In cardiomyocytes, 2 classes of guanylyl cyclases (GCs) are responsible for cGMP synthesis. The first class, particulate GCs (pGCs) represented by GC-A and GC-B, are plasma membrane receptors for atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP), respectively.^{3,4} Second, the so-called soluble or NO-sensitive GCs (NO-GCs) are also functionally present in cardiomyocytes.^{5–7} cGMP levels are negatively regulated by cGMP-hydrolyzing enzymes phosphodiesterases (PDEs) with at least 4 families (PDE1, 2,

3, and 5) expressed in cardiac myocytes, whereby the first 3 PDEs can degrade both cGMP and cAMP.^{8–11} cGMP is generally considered as a cardioprotective second messenger,¹² because pharmacological elevation of cGMP levels either by inhibition of cGMP-hydrolyzing PDE5 and PDE1 or by activation of NO-GC and pGC prevents pathological cardiomyocyte growth in vitro¹³ and cardiac remodeling in vivo.^{2,14–16}

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Reliable cGMP measurements in adult cardiomyocytes have been challenging.¹⁷ In adult heart, cGMP is present at much lower concentrations than cAMP and acts in a compartmentalized

Original received August 21, 2013; revision received February 10, 2014; accepted March 4, 2014. In February 2014, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 13.8 days.

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The online-only Data Supplement is available with this article at <http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.114.302437/-/DC1>.

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DOI: 10.1161/CIRCRESAHA.114.302437

Nonstandard Abbreviations and Acronyms

ANP	atrial natriuretic peptide
cGMP	3',5'-cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
FRET	Förster resonance energy transfer
IBMX	3-isobutyl-1-methylxanthine
ISO	isoproterenol
NO-GC	NO-sensitive guanylyl cyclase (GC)
ODQ	1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one
PDE	phosphodiesterase
pGC	particulate GC
RIA	radioimmunoassay
SNAP	S-nitroso-N-acetyl-D,L-penicillamine
TAC	transverse aortic constriction

fashion,^{1,8} so that robust techniques to monitor this second messenger with high sensitivity and temporal and spatial resolution are desirable. During the past decade, several optical and nonoptical methods to measure cGMP in single intact cells have been developed. Electrophysiological recordings using ectopically expressed cyclic nucleotide gated channels as sensors for subsarcolemmal cGMP have uncovered differential contributions of various PDE families in compartmentation of cGMP in adult rat ventricular myocytes. In particular, GC-A–derived cGMP is exclusively controlled by PDE2, whereas PDE5 exclusively controls cGMP pools produced by NO-GC under physiological conditions.¹⁸ However, this technique has certain limitations such as restriction of the sensor to the subsarcolemmal compartment and relatively low cGMP/cAMP selectivity.¹⁹ In parallel, several fluorescent cGMP biosensors have been developed that allow visualization of intracellular cGMP with high temporal and spatial resolution.²⁰ Most of these sensors are based on a partially truncated cGMP-dependent protein kinase molecule that changes its conformation on cGMP binding, thereby leading to a change in Förster resonance energy transfer (FRET) between cyan and yellow fluorescent proteins fused to the kinase backbone,^{21–23} or to an increase in fluorescence of circularly permuted green fluorescent protein attached to its C-terminus.²⁴ These sensors represent a major advance in cGMP imaging, but have either relatively low sensitivity ($\approx 1 \mu\text{mol/L}$ affinity for cGMP, which is ≈ 5 – 10 times less than the affinity of cGMP-dependent protein kinase) or, again, very low cGMP/cAMP selectivity. These sensors were successfully used to uncover compartmentalized cGMP signaling in neonatal rat cardiomyocytes,^{25,26} but failed to robustly report cGMP in adult myocytes that produce much less cGMP.

Recently, a low-affinity biosensor²⁷ based on a single regulatory cGMP-binding domain from PDE5 has been modified by exchanging cyan and yellow with green (T-Sapphire) and red (Dimer2) fluorescent proteins. Unexpectedly, this modification led to ≈ 40 -fold increase in the affinity for cGMP (40 nmol/L versus $1.5 \mu\text{mol/L}$) together with the retained low affinity for cAMP ($>1 \text{ mmol/L}$),²⁸ making this biosensor (called red cGES-DE5) promising for cGMP measurements in adult cardiomyocytes. Here we transgenically expressed this biosensor in adult mouse ventricular myocytes and showed that in these cells basal cGMP levels are as low as $\approx 10 \text{ nmol/L}$, can be markedly stimulated by pGC ligands, especially by the

CNP, and that they are dynamically regulated by the activities of NO-GC and several PDE families. We think that this new transgenic cGMP-FRET sensor mouse should become a widespread tool for the visualization of cGMP in adult cardiomyocytes under physiological and pathophysiological conditions.

Methods

The expanded Methods section is available in the Online Data Supplement.

Transgenic Mice

DNA encoding the red cGES-DE5 sensor was excised from the original pcDNA3.1-based vector²⁸ and subcloned via KpnI and XhoI restriction sites into the previously described vector containing the α -myosin heavy chain promoter and polyadenylation signal.²⁹ The resulting vector was linearized with SpeI, purified, and used for pronuclear injections to generate transgenic mice on the FVB/NRj background (obtained from Janvier Laboratories, Saint Berthevin, France) as described.³⁰ All the procedures involving mice were in accordance with institutional and governmental guidelines.

FRET Measurements

Single adult mouse ventricular cardiomyocytes were freshly isolated and subjected to FRET measurements as previously described.³¹ Red cGES-DE5 was excited with 405 nm light from Polychrome V (Till Photonics). Emission light was split into donor and acceptor channels using the DV2 QuadView (Photometrics) equipped with the 565dcxr dichroic mirror and BP515/30 and BP590/40 emission filters. Changes in FRET were monitored at room temperature on bath application of various ligands using VisiView imaging software (Till Photonics). The raw data were corrected offline for the bleedthrough of the donor into the acceptor channel and for photobleaching as described.³¹

Transverse Aortic Constriction

Mice 9 to 13 weeks old were randomized to sham or transverse aortic constriction (TAC) group. Mice were anesthetized using 1.5% to 2% isoflurane in pure O₂. A suprasternal incision was made and the aortic arch visualized using a binocular macroscope (Olympus). TAC³² occurred by spacer-defined (26-gauge) constriction using a 6-0 polyvinylene suture between the first and second trunk of the aortic arch. For sham, the aorta was not constricted. Three days after surgery, Doppler velocity was measured by a 20 MHz probe to quantify the pressure gradient across the TAC or after sham procedure by transthoracic echocardiography (VisualSonics; Vevo 2100, Toronto, Canada). Mice received analgesic therapy with metamizole 3 days before and for 1 week after surgery. Mice were analyzed by echocardiography and euthanized 8 to 9 weeks after surgery for ventricular cardiomyocyte isolation and single-cell microscopy.

Statistics

Morphometric, echocardiographic, working heart, radioimmunoassay (RIA), and FRET data were analyzed using Origin Pro 8.6 software (OriginLab Corporation, Northampton, MA) and presented as mean $\pm$ SE from the indicated number of independent experiments (animals) per condition. Numbers of mice and single cells are indicated in figure legends and above the bars in the graphs. Raw data were analyzed for normal distribution using the Kolmogorov–Smirnov test. When normally distributed, data were compared using mixed ANOVA followed by χ^2 test, or 1-way ANOVA for simple 2-group comparison. Otherwise, nonparametric tests (Kruskal–Wallis followed by Dunns test or Mann–Whitney) were applied. Differences were considered significant at $P < 0.05$.

Results

Generation of Red cGES-DE5 Transgenic Mice

To be able to measure cGMP levels in freshly isolated adult cardiomyocytes, we generated transgenic mice by pronuclear injection of a construct that expresses this biosensor under the control

of the α -myosin heavy chain promoter (Figure 1A). Two PCR-positive founders were obtained, 1 of which bred normally and gave rise to a phenotypically normal line of offspring. Cardiac morphology and heart function in these mice were not affected by sensor expression, as demonstrated by histological, morphometric, and echocardiographic analysis performed at the age of 6 months (Figure 1B–1D; Online Table I). The heart to body weight ratios were also not changed at 3 months of age (4.9 ± 0.1 for both wild-type and transgenic mice; $n=12$ –14), as were the heart weight to tibia length ratios (68.6 ± 2.4 and 68.1 ± 1.2 for wild-type and transgenic mice, respectively; $n=12$ –14). There was also no difference in cell size, as measured in WGA-stained transverse heart sections (Figure 1E). Next, we isolated adult cardiomyocytes from these mice and observed strong green and red cytosolic fluorescence in T-Sapphire and Dimer2 channels in all transgenic cells (Figure 1F).

FRET Measurements of cGMP Synthesis and Degradation

To analyze cGMP levels in real time, we used freshly isolated transgenic ventricular myocytes and stimulated them with ligands that activate NO-GC, GC-A, and GC-B. Interestingly, FRET signals induced by NO donors, such as sodium nitroprusside (Online Figure I) and

S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), were not present in every cell (Figure 2A and 2E). Even NO donors with faster kinetics of release such as diethylamine NONOate and PROLI NONOate did not evoke any signal (Online Figure I). However, some basal NO-GC activity was clearly present (Figure 6), and SNAP application after combined prestimulation with β -adrenergic receptor agonist and PDE5 inhibitor tadalafil showed small but clear FRET responses (see below). In contrast, pGC ligands at saturating concentrations increased cGMP, although with variable amplitudes but to much higher levels, especially the GC-B agonist CNP that evoked significantly stronger cGMP responses than the GC-A ligand ANP (Figure 2B–2E; Online Figure II). The absence of sensor response to NO donors and negligible response to ANP were unlikely the result of improper sensor localization, because in neonatal rat cardiomyocytes, which express ≈ 3 -fold higher levels of NO-GC, red cGES-DE5 showed clear responses to SNAP and ANP (Online Figure III), which exactly reflected the behavior of these cells reported previously using other FRET biosensors.^{6,25} Quantification of basal and stimulated cGMP levels in adult mouse myocytes using RIA performed in the presence of 3-isobutyl-1-methylxanthine (IBMX) revealed comparable magnitudes of responses as measured by FRET in the presence of IBMX (Figure 2F), suggesting that

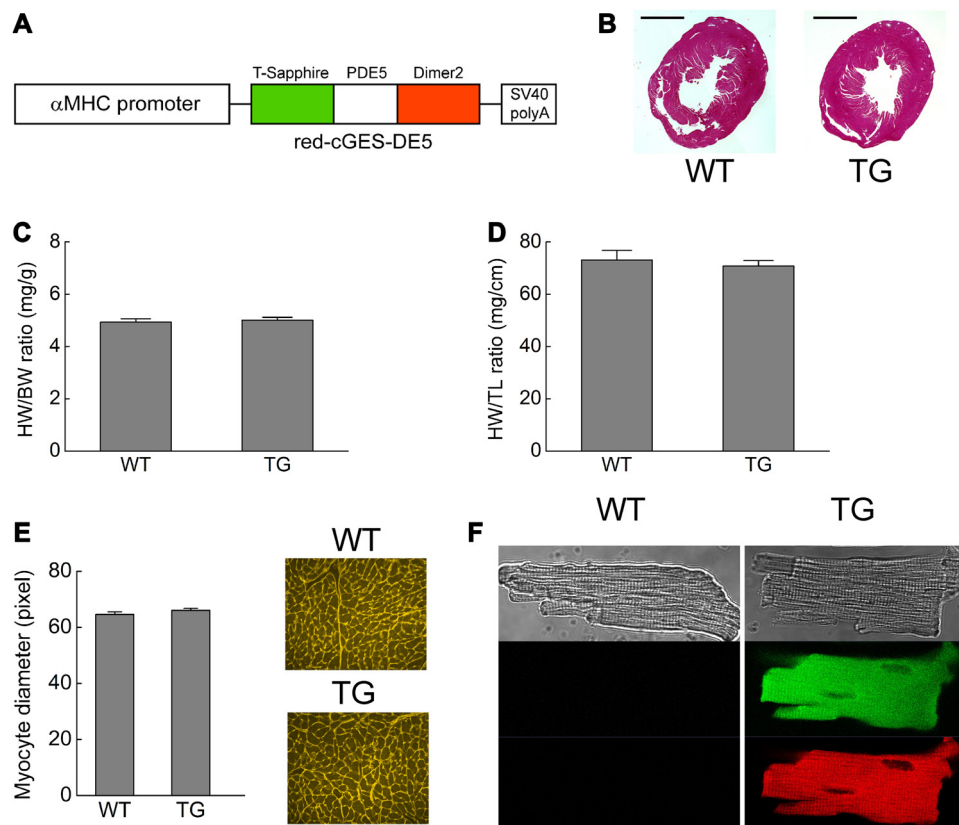


Figure 1. Characterization of red cGES-DE5 transgenic mice. **A**, The sensor sequence, including 2 fluorescent proteins and the 3',5'-cyclic guanosine monophosphate-binding site from phosphodiesterase (PDE)5, was expressed under the control of cardiac muscle-specific α -myosin heavy chain (α MHC) promoter. Schematic of the transgenic construct is shown. **B**, H&E stainings of representative cross-sections from wild-type (WT) and transgenic (TG) hearts at the age of 6 mo. Scale bar, 2 mm. Morphometric analysis of heart weight to body weight (HW/BW; **C**) and heart weight to tibia length (HW/TL) ratios (**D**; mean $\pm$ SE; $n=11$ –22 each) at the same age does not show any abnormalities in transgenic mice. **E**, Analysis of cardiomyocyte diameter in transverse heart sections stained with WGA. Data are mean $\pm$ SE from 3 WT and 5 TG hearts with 100 cells counted in each section. **F**, Confocal images of a WT and TG cardiomyocyte in T-Sapphire and Dimer2 channels. TG cells show a robust cytosolic expression of the sensor.

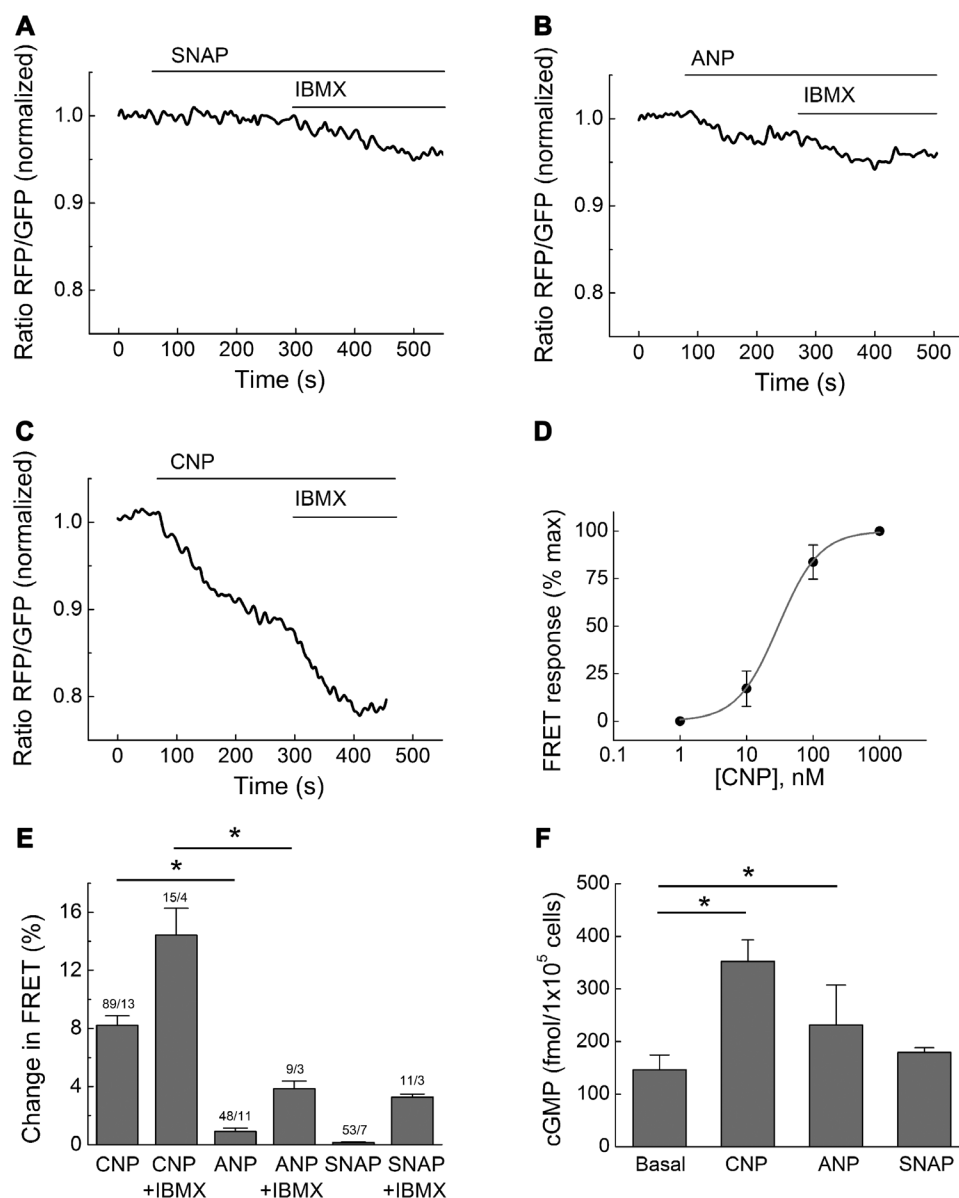


Figure 2. Measurements of NO-sensitive guanylyl cyclase (NO-GC), GC-A, and GC-B activity. Representative single-cell recordings from transgenic myocytes stimulated with either 100 μ mol/L S-nitroso-N-acetyl-D,L-penicillamine (SNAP; **A**), 100 nmol/L atrial natriuretic peptide (ANP; **B**), or 100 nmol/L C-type natriuretic peptide (CNP; **C**), and subsequently with 100 μ mol/L 3-isobutyl-1-methylxanthine (IBMX) to block phosphodiesterase activity. Decrease in the Dimer2/T-Sapphire FRET ratio (designated as RFP/GFP ratio) corresponds to an increase in intracellular 3',5'-cyclic guanosine monophosphate (cGMP). **D**, Concentration-response dependence of CNP effect ($n=8$ cells from 3 mice). **E**, Quantification of FRET responses from **A** to **C**. Data were from the time points at a clear plateau of each response (mean $\pm$ SE). The numbers of mice and cells analyzed per condition are shown above the bars (as n cells/ N mice). **F**, Measurements of cGMP levels using radioimmunoassay in adult mouse myocytes. Cells were stimulated for 10 min with 100 nmol/L ANP, 100 nmol/L CNP, or 100 μ mol/L SNAP in the presence of IBMX. Bars show mean $\pm$ SE from 3 to 4 mice per condition. Significant differences ($P<0.05$) are indicated with asterisks.

the sensor properly reflects overall changes of cGMP in the cytosolic compartment of adult mouse cardiomyocytes.

To study whether the cGMP-FRET data acquired in single isolated cells correlate with cGMP levels and cGMP-dependent protein kinase activity in intact beating hearts, we stimulated working hearts the same way and performed cGMP-RIA and immunoblot analysis of cardiac cGMP-dependent protein kinase substrate phospholamban³³ phosphorylation in left ventricular tissue. These experiments showed a robust effect of CNP and a slight stimulatory effect of SNAP on cGMP levels but not on PLN phosphorylation (Figure 3). Interestingly,

ANP did not affect cGMP levels, which was in contrast to previously observed ≈ 2.5 -fold increase in C57BL/6;129 mice under the same conditions.³⁴ This might argue for the differences of GC-A expression between various genetic backgrounds.

Next, we tested the contribution of various PDEs involved in cGMP degradation at basal state by the use of selective PDE1, 2, 3, and 5 inhibitors. The PDE1 inhibitor 8-methoxymethyl-3-isobutyl-1-methylxanthine applied alone had no effect on basal cGMP levels (Figure 4A). Unexpectedly, cGMP levels were increased by the selective PDE3 inhibitor cilostamide, whereas the selective PDE2 and PDE5 inhibitors

BAY-60-7550 and tadalafil, respectively, showed only negligible effects (Figure 4B–4D). The effect of cilostamide at 10 $\mu\text{mol/L}$ was as strong as that of the unselective PDE inhibitor IBMX applied alone (100 $\mu\text{mol/L}$; Figure 4E). Clear effects of cilostamide and IBMX alone also indicated that there is a source of basal cGMP production.

An important advantage of transgenic sensor mice is the possibility of combining them with various genetic and experimental disease models. To this end, we studied whether the relative contributions of different PDEs might be changed in cardiac disease and performed TAC to induce a mild compensated pressure overload cardiac hypertrophy (Online Table II). We isolated cardiomyocytes from TAC and sham-operated mice to perform similar experiments as shown in Figure 4E. Interestingly, although there was no difference in maximal CNP plus IBMX response ($11.6 \pm 1.4\%$ and $10.3 \pm 1.3\%$ in TAC versus sham; $n=24$ cells from 4 to 6 mice), we could observe a significant increase of PDE5 inhibitor effect (Figure 4F). In contrast to previously described upregulation of PDE1 and downregulation of PDE3 activities under more intense hypertrophy conditions,^{15,35} the responses to PDE1 and PDE3 inhibitors were unchanged in our mild TAC model (Figure 4F).

Next, we studied the role of individual PDE families in the hydrolysis of cGMP under stimulated conditions, such as occurring in intact heart and in disease. We first pretreated the cells with a low dose of isoproterenol (ISO) to mimic physiological catecholamine-stimulated cAMP concentrations or with angiotensin II in combination with bone natriuretic peptide to mimic pathological conditions and then inhibited PDEs (Figure 5A and 5B). Under these conditions, which are more close to the situation in intact heart, cilostamide effects were still present, suggesting that PDE3 continued to play its important role in cGMP hydrolysis. In the presence of competitive substrate cAMP, cilostamide effects were reduced by $\approx 50\%$ but still remained significantly stronger than those of other PDE inhibitors (Figure 5A). Likewise, after prestimulation

with CNP (Figure 5C), ANP, or SNAP, strong cilostamide effects were unchanged (Online Figure IV).

Basal cGMP Levels Are Low in Adult Cardiomyocytes

To estimate absolute basal cGMP concentrations in the cytosol of adult mouse myocytes and to identify the source of basal cGMP production, we measured FRET responses to the NO-GC inhibitor 1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one (ODQ), which should decrease cGMP levels to minimal levels measurable by the sensor. Within a few minutes, ODQ 50 $\mu\text{mol/L}$ led to an increase of FRET ratio by $4.0 \pm 0.7\%$, which is indicative of a decrease of basal cGMP. The response to IBMX after ODQ application was virtually abolished (Figure 6A and 6B), whereas the pGC inhibitor A71915 showed no effect on FRET ratio (Figure 6B), suggesting that the NO-GC activity is primarily responsible for basal cGMP production. When compared with maximal FRET responses evoked by CNP plus IBMX ($14.4 \pm 1.9\%$; Figure 2E) and the concentration–response curve of red cGES-DE5 sensor protein to cGMP measured *in vitro*,²⁸ basal cGMP levels in adult mouse cardiomyocytes can amount to ≈ 10 nmol/L.

PDE3 Is Involved in cGMP/cAMP Crosstalk

cGMP has been shown to affect cardiomyocyte cAMP levels in 2 ways. It can either decrease cAMP via PDE2 activation or increase it via PDE3 inhibition,⁹ which in neonatal cardiomyocytes depends on the studied cAMP microdomain.²⁵ We wondered which of these mechanisms plays the predominant role after β -adrenergic stimulation in the cytosol of adult mouse cardiomyocytes. To answer this question, we used ventricular cardiomyocytes isolated from mice transgenically expressing the cAMP sensor Epac1-camps.³⁶ These cells react to the β -adrenergic receptor agonist ISO with an increase of cAMP, which is visualized by a decrease of FRET. Subsequent application of CNP or, to a smaller extent, ANP led to a further

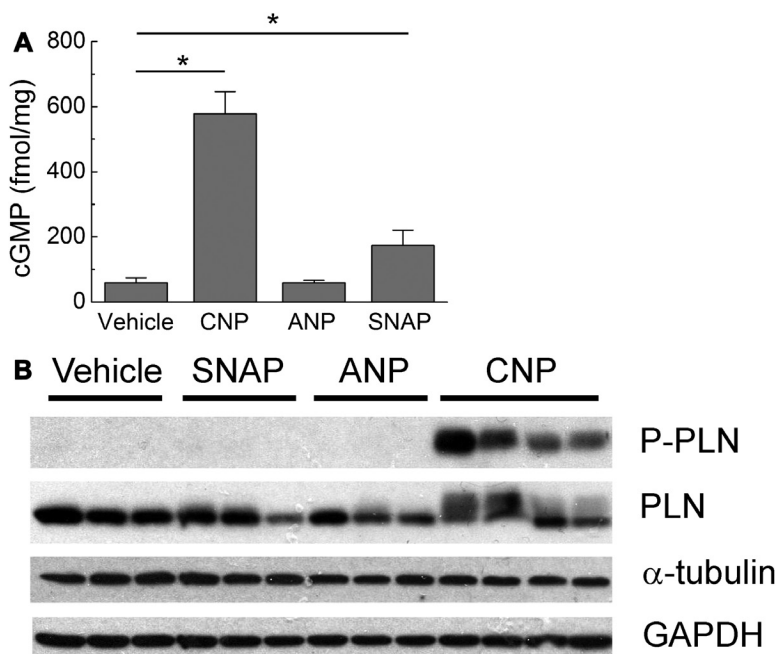


Figure 3. Measurements of stimulated 3',5'-cyclic guanosine monophosphate (cGMP) levels and cardiomyocyte cGMP-dependent protein kinase (cGK) activity in working hearts. Intact working hearts were perfused for 10 minutes with vehicle, C-type natriuretic peptide (CNP; 100 nmol/L), atrial natriuretic peptide (ANP; 100 nmol/L), or S-nitroso-N-acetyl-D,L-penicillamine (SNAP; 100 $\mu\text{mol/L}$); left ventricles were dissected and processed for cGMP radioimmunoassay (**A**). Shown are mean $\pm$ SE; $n=4$ to 5 hearts each. **B**, Immunoblot analysis of cGK substrate phospholamban phosphorylation (P-PLN) in the same heart tissue samples. Total phospholamban (PLN), α -tubulin, and GAPDH were used as a loading control.

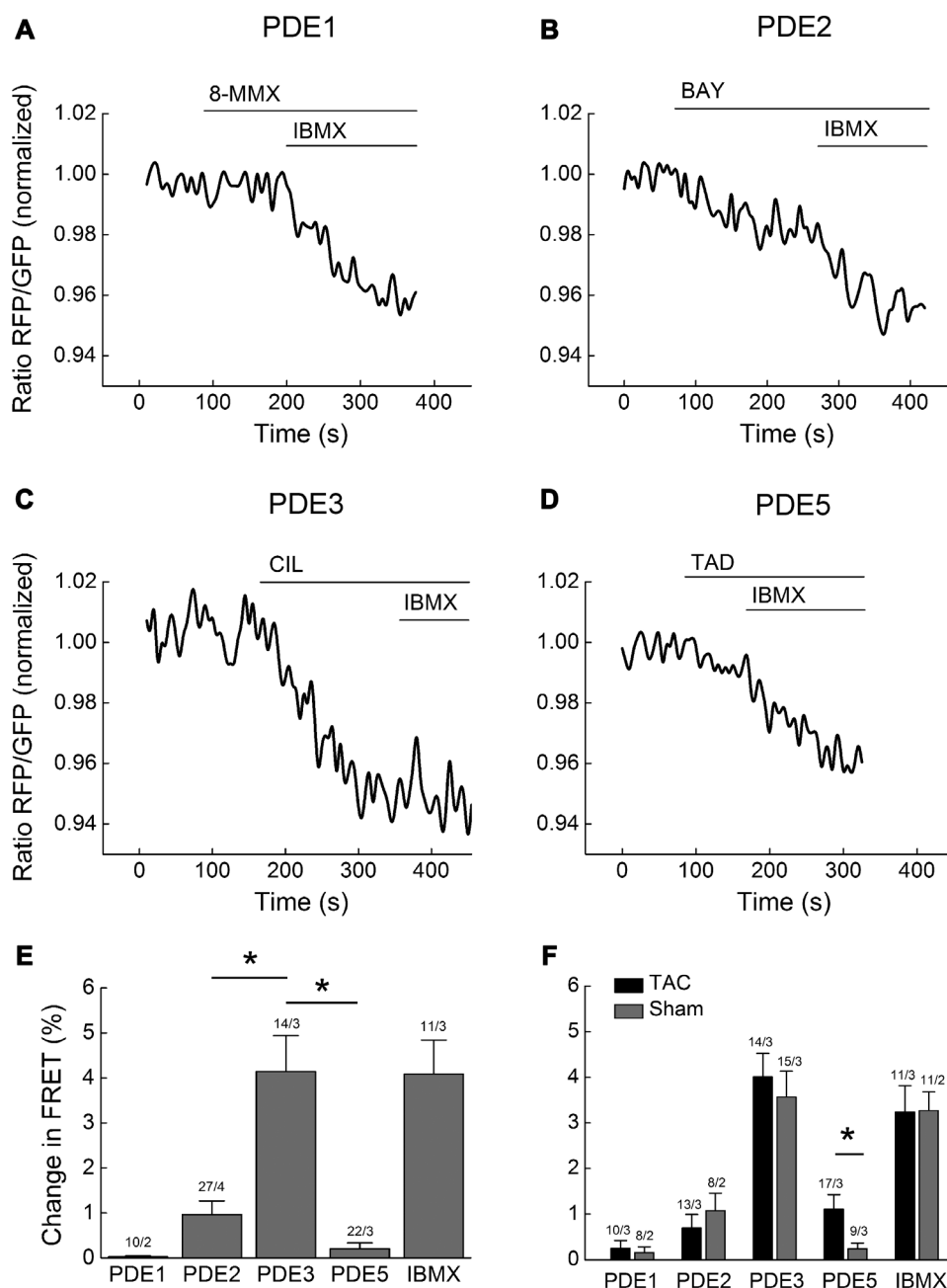


Figure 4. Analysis of basal 3',5'-cyclic guanosine monophosphate (cGMP) degradation by various cGMP-phosphodiesterases (PDEs). Transgenic cardiac myocytes were treated with selective PDE inhibitors and subsequently with the unselective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX). 8-Methoxymethyl-3-isobutyl-1-methylxanthine (8-MMX; 10 μ mol/L) to block PDE1 (**A**), 100 nmol/L BAY-60-7550 (BAY) to inhibit PDE2 (**B**), 10 μ mol/L cilostamide (CIL) for PDE3 (**C**), and 100 nmol/L tadalafil (TAD) for PDE5 were used (**D**). **E**, Quantification of Förster resonance energy transfer (FRET) responses from **A** to **D** and of the IBMX effect when applied alone. RFP/GFP ratio indicates Dimer2/T-Sapphire FRET ratio. **F**, Analysis of FRET responses to PDE inhibitors in cardiomyocytes isolated from transverse aortic constriction (TAC) and sham mice. The numbers of mice and cells analyzed per condition (**F** and **E**) are shown above the bars (as n cells/N mice). Significant differences ($P<0.05$) are indicated with asterisks.

significant increase in cAMP levels (Figure 7A and 7B). In contrast, the application of 100 nmol/L CNP alone did not lead to any significant response ($0.38\pm0.38\%$ of ISO; $n=13$ cells from 2 hearts). The PDE3 inhibitor cilostamide increased cAMP after ISO (Figure 7E and 7F), whereas the inhibition of PDE2 with BAY-60-7750 could not further raise intracellular cAMP after CNP application ($115.9\pm4.4\%$; $n=9$ cells from 3 mice), suggesting that after β -adrenergic stimulation, the increase in intracellular cGMP leads to an increase of cytosolic

cAMP levels due to PDE3 inhibition. The stimulatory effects of ANP and CNP after ISO were abolished in cells preincubated with cilostamide, confirming the central role of PDE3 in this type of cGMP/cAMP crosstalk (Figure 7C, 7D, and 7F). This finding suggests that in isolated adult mouse cardiomyocytes, PDE3 is involved in controlling the natriuretic peptide-stimulated cGMP pools responsible for cGMP/cAMP crosstalk. Interestingly, pretreatment of cells with the PDE5 inhibitor tadalafil did not significantly affect ANP and CNP

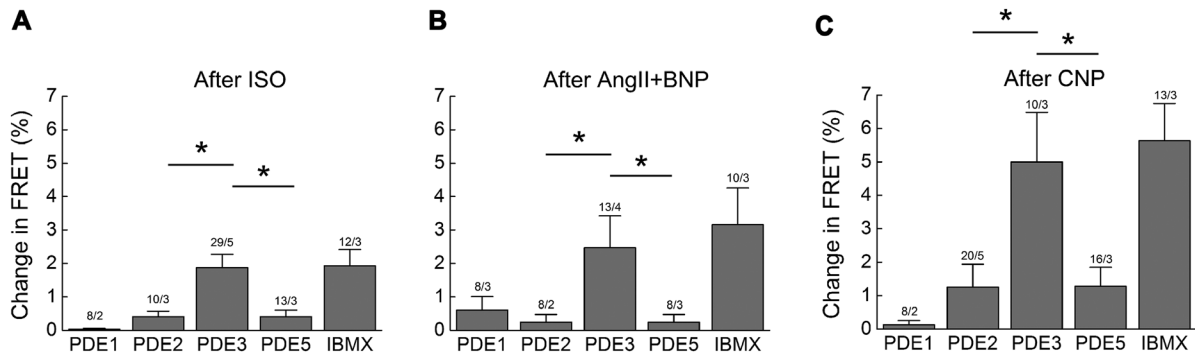


Figure 5. Analysis of phosphodiesterase (PDE) inhibitor effects on 3',5'-cyclic guanosine monophosphate (cGMP) levels at prestimulated state. Cardiomyocytes were first prestimulated for 5 min with 0.1 nmol/L isoproterenol (ISO; **A**), with a combination of 100 nmol/L angiotensin II (AngII) plus 100 nmol/L bone natriuretic peptide (BNP) to mimic neurohormonal stimulation in disease (**B**), or with 100 nmol/L C-type natriuretic peptide (CNP; **C**) to increase cGMP levels, and PDE1, 2, 3, and 5 inhibitors were applied as described in Figure 4A–4E to measure changes in Förster resonance energy transfer (FRET) ratio evoked by each PDE inhibitor. The numbers of mice and cells per condition are shown above the bars (as n cells/N mice). Significant differences ($P < 0.05$) are indicated with asterisks.

responses but strongly augmented the response to SNAP (Figure 7F). Therefore, we monitored cGMP levels under the same stimulatory conditions. Combined application of TAD and SNAP after ISO now led to measurable FRET responses ($3.7 \pm 0.4\%$; $n = 12$ cells from 3 mice), suggesting that the transgenic sensor is capable of detecting the cGMP pool, which is produced by NO-GC after β -adrenergic stimulation and controlled by PDE5.

Discussion

Initially, we generated transgenic mice with an older low-affinity ($\approx 0.9 \mu\text{mol/L}$) cGMP biosensor cGES-DE2 but could not detect any change in FRET on NO-GC and pGC stimulation in adult cardiac myocytes. Therefore, we hypothesized that only a highly sensitive FRET sensor can be used for cGMP imaging in these cells, whereas other biosensors, including cGi500 (affinity $\approx 0.5 \mu\text{mol/L}$), for which an ubiquitous transgenic mouse has recently been published,³⁷ would not pick up low cGMP levels typical for adult cardiomyocytes. Here we generated transgenic mice expressing a highly sensitive cGMP biosensor red cGES-DE5 and used them for FRET measurements. To our knowledge, this is the first report on real-time FRET-based cGMP imaging in adult mouse cardiomyocytes.

Constitutive transgenic expression of the sensor in mouse hearts did not lead to any phenotypic abnormalities as shown by morphometric analysis and echocardiography (Figure 1; Online Table I). The sensor was uniformly expressed in the cytosol of all cardiomyocytes at a high enough level to allow reliable FRET measurements. Interestingly, most of the cells did not respond to NO-GC stimulation by NO donors, suggesting that NO-GC is neither highly expressed in these cells nor accessible to bioactive NO generated by these substances in cardiac myocytes. Indeed, previous reports in adult mouse cardiomyocytes suggested a relatively low NO-GC expression³⁸; these cells expressed ≈ 3 -fold less NO-GC compared with neonatal cardiomyocytes (Online Figure IIB); and the effects of exogenous NO donors in ventricular (contrary to atrial) myocytes have been inconsistent. In addition, high concentrations of myoglobin might partially prevent NO-dependent effect due to its scavenging.³⁹ Another possibility is that the concentration of the cytosolic sensor in NO-GC-associated microdomain is not high enough to monitor cGMP on direct stimulation. However, we were able to observe a clear effect of SNAP in neonatal rat cardiomyocytes (Online Figure III) and absence of any effect in RIA measurements (Figure 2F), which would argue against improper

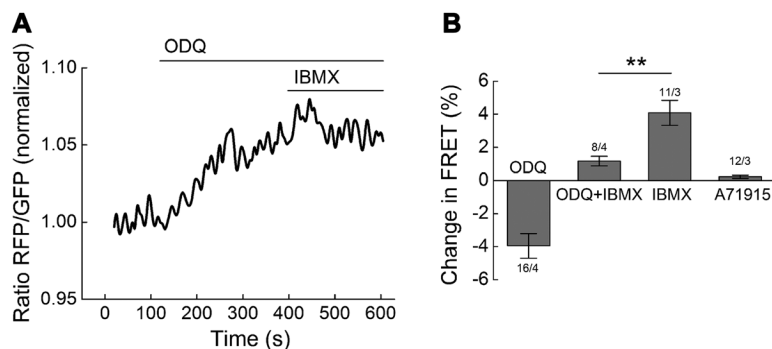


Figure 6. Analysis of basal NO-sensitive guanylyl cyclase (NO-GC) and particulate GC (pGC) activity. **A**, Red cGES-DE5 expressing myocytes were treated with the NO-GC inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ; 50 $\mu\text{mol/L}$), which decreases basal 3',5'-cyclic guanosine monophosphate levels and blocks the subsequent 3-isobutyl-1-methylxanthine (IBMX) response. RFP/GFP ratio indicates Dimer2/T-Sapphire FRET ratio. **B**, Quantification of Förster resonance energy transfer (FRET) responses to ODQ shown in **A**, and the responses to the pGC inhibitor A71915 (1 $\mu\text{mol/L}$), which did not affect the FRET ratio (mean $\pm$ SE). The numbers of mice and cells per condition are shown above the bars (as n cells/N mice). The IBMX bar is from Figure 4E to facilitate comparison. ** Indicates significant differences at $P < 0.01$.

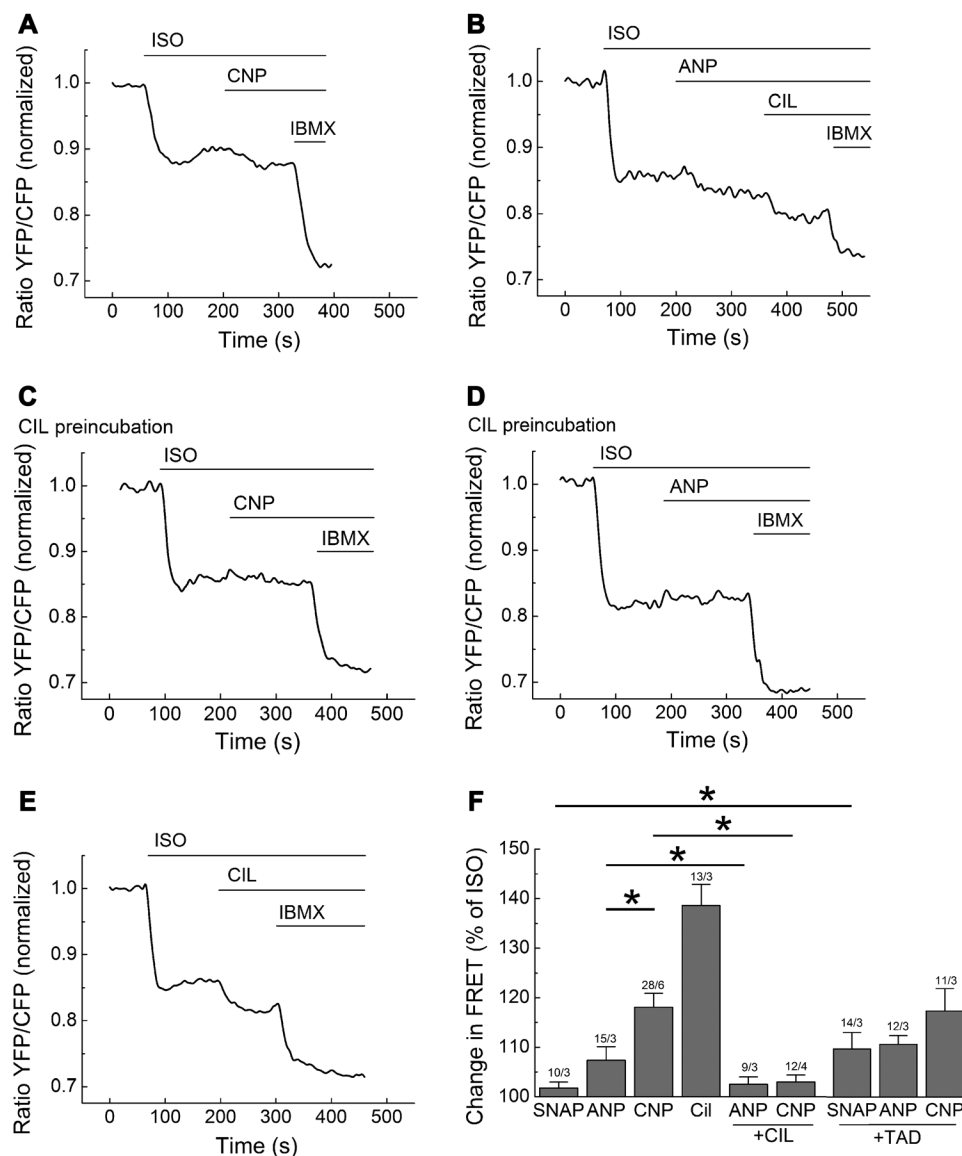


Figure 7. Phosphodiesterase (PDE)3-mediated 3',5'-cyclic guanosine monophosphate/cAMP crosstalk. Cardiomyocytes transgenically expressing the cAMP sensor Epac1-camps were stimulated with the β -adrenergic agonist isoproterenol (ISO; 100 nmol/L) and subsequently with 100 nmol/L C-type natriuretic peptide (CNP; **A**) or 100 nmol/L atrial natriuretic peptide (ANP; **B**), which further increased cAMP levels. Decrease in Förster resonance energy transfer (FRET) corresponds to an increase in intracellular cAMP. Pretreatment with 10 μ mol/L of the PDE3 inhibitor cilostamide (CIL; 3 min) prevented the CNP (**C**) and ANP (**D**) effects on ISO-stimulated cAMP levels. **E**, Cells were stimulated first with ISO and subsequently with 10 μ mol/L CIL, which further increased cAMP (positive control). **F**, Quantification of the FRET responses shown in **A** to **E**, as well as responses to peptides or S-nitroso-N-acetyl-L-penicillamine (SNAP; 100 μ mol/L) applied after ISO with or without 100 nmol/L tadalafil (TAD); mean $\pm$ SE. The numbers of mice and cells per condition are shown above the bars (as n cells/N mice). Significant differences ($P<0.05$) are indicated with asterisks.

localization of the sensor. In contrast, previously published data documented small but significant increases of cardiomyocyte cGMP after NO donor stimulation in C57BL/6 myocardium,⁴⁰ which was also the case in our working heart experiments. We also tested the possibility that the isolation procedure might lead to oxidation and loss of NO sensitivity of NO-GC. Even when all isolation steps and measurements were performed in the presence of the reactive oxygen species scavenger melatonin (100 μ mol/L),⁴¹ no response to NO donors could be attained. In addition, the direct NO-GC activator BAY-58-2667 (cinaciguat) capable of activating even oxidized NO-GC⁴² did not show any response (Online Figure I), suggesting that cardiomyocyte isolation per se is unlikely

to inactivate NO-GC. However, under combined ISO and TAD treatment, NO donor effects could be unmasked, which supports the previously established stringent compartmentation of NO-GC/cGMP pools by PDE5 after β -adrenergic stimulation (Online Figure VI).⁶ Remarkably, we could observe clear effects of the NO-GC inhibitor ODQ (Figure 6), which suggest that constitutively active NO-GC is present in these cells. Because the NO-GC-associated cGMP pools and the proper localization of this enzyme are particularly important for heart function and pathophysiology,⁴⁰ it will be intriguing to generate targeted versions of red cGES-DE5 (eg, targeted to the plasma membrane) to dissect cGMP signaling inside the membrane signalosomes. The advantage of

transgenic mice expressing a FRET biosensor is the possibility of combining them with experimental mouse models for hypertrophy and heart failure with subsequent analysis of cGMP in freshly isolated cells, as demonstrated by measurements in TAC myocytes (Figure 4F).

In contrast to less active NO-GC, cGMP levels could be strongly stimulated by CNP (Figure 2C–2E). Both ANP and CNP receptors GC-A and GC-B have been described in cardiac myocytes, but it is still controversial which one of these GCs plays a predominant role.^{4,43,44} Here we found that GC-B activation by CNP leads to much more pronounced cytosolic cGMP signals, suggesting that this receptor might be particularly important for the regulation of cardiomyocyte cGMP. This is in line with the published and whole-heart functional data showing stronger CNP effects on contractility and phospholamban phosphorylation when compared with ANP (Figure 3; Online Figure VI).^{33,34}

The constitutive NO-GC activity is balanced by the basal activity of PDEs involved in cGMP degradation (Figure 4). Unexpectedly, PDE3 was found to regulate cytosolic cGMP levels, whereas PDE2 and PDE5 showed only minor effects. The latter isoforms might be particularly important for local control of cGMP in subcellular compartments, as suggested in earlier studies.^{6,18,45} PDE3 can degrade both cAMP and cGMP. It is often referred to as the cGMP-inhibited PDE, that is, cGMP can block cAMP degradation by PDE3, and thought to be mainly responsible for cAMP hydrolysis, because the $V_{\max}$ values for cAMP hydrolysis are almost 10-fold higher than for cGMP as a substrate.^{46,47} Likewise, cAMP can inhibit the cGMP–PDE activity of PDE3. Indeed, the presence of cAMP as the competitive substrate in cardiomyocytes, stimulated with a low dose of the β -adrenergic agonist ISO to mimic physiological catecholamine levels present in the heart (Figure 5A), led to a reduction of the cilostamide effect on intracellular cGMP measured by $\approx 50\%$. However, even in this case, PDE3 contribution remained important. PDE activity profile measured by FRET does not exactly match the data from in vitro PDE activity assay performed with cardiomyocyte lysates (Online Figure V). Because this assay is routinely done at very high substrate concentrations, such as 1 $\mu\text{mol/L}$ cGMP, these in vitro conditions are vastly different from physiological low cGMP values in cardiomyocytes (by the factor of 100), possibly leading to a decrease of PDE3 activity and underestimation of its contribution. Our FRET approach works in intact cells at endogenous cGMP levels and provides a more sensitive and more physiological way to analyze PDE activity. At this point, it is worth mentioning again that we measured PDE inhibitor effects in the whole cytosolic compartment. Next, it will be interesting to do it in distinct subcellular microdomains, which is only possible with localized FRET sensors, but not with the classical biochemical assays. Therefore, we think that, compared with classical assays, our approach is more precise because it better reflects the behavior of intact cells.

PDE3 has been previously characterized as a PDE, which, together with PDE2, is crucial for the cGMP/cAMP crosstalk in neonatal rat cardiomyocytes.^{9,25} We studied this mechanism in the cytosol of adult mouse myocytes after β -adrenergic stimulation and found that cAMP elevation via cGMP-dependent

PDE3 inhibition after CNP plays a predominant role in the cytosolic compartment of adult cardiomyocytes (Figure 7; Online Figure VI). This mechanism might account for the initial increase in cardiac contractility observed in isolated hearts on CNP infusion,³⁴ as well as the positive inotropic effect of CNP in failing cardiomyocytes.⁴⁸ Moreover, this type of PDE3-dependent cGMP/cAMP crosstalk, together with upregulation of PDE5 activity, has been shown to play an important role for the regulation of contractility in hypertrophied myocardium.⁴⁹ To further study cAMP/cGMP crosstalk, it is possible to generate double transgenic mice that express both sensors, the red cGES-DE5 sensor and Epac1-camps, to visualize cGMP and cAMP dynamics simultaneously.²⁸

In general, in vitro cGMP–PDE assays are not easy to perform at low substrate concentrations, and RIAs are usually done under complete PDE inhibition with IBMX. Therefore, our live cell FRET approach is a powerful way of analyzing the role of various PDEs in the regulation of physiologically low basal cGMP levels in intact adult cardiomyocytes. In addition, FRET measurements allow real-time monitoring and detailed kinetic analysis of responses to various pharmacological GC ligands and PDE inhibitors alone or in combination, in healthy and diseased adult cardiomyocytes. An important limitation of the method is the use of single isolated myocytes, which are not always directly comparable to the cells in intact myocardium. The cell isolation process might induce significant cellular stress and generation of oxidative species that can potentially affect the activities of GCs and PDEs. However, the rapid development of imaging techniques should soon allow FRET measurements to be performed in intact hearts for which the presented robust transgenic expression of the biosensor is indispensable. In conclusion, we think that our newly generated cGMP sensor mice will find a broad application to study cardiac cGMP signaling.

Acknowledgments

We thank the transgenic core unit of the Max Plank Institute for Experimental Medicine for transgenic mouse generation; Katharina Völker and Birgit Gassner for working heart and RIA experiments; Kirsten Koschel for echocardiography; as well as Karina Zimmermann and Tobias Goldak for excellent technical assistance.

Sources of Funding

This work was supported by the Deutsche Forschungsgemeinschaft (grant NI 1301/1-1 and FOR2060 to V.O.N., SFB 1002 to V.O.N. and S.E.L., KFO155 to S.E.L., and SFB688 to M.K.), the British Heart Foundation (RG/12/18/30088 to J.G.), and the University of Göttingen Medical Center (pro futura grant to V.O.N.).

Disclosures

None.

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Novelty and Significance

What Is Known?

- 3',5'-Cyclic guanosine monophosphate (cGMP) is an important second messenger, which under certain conditions protects the heart from hypertrophy and heart failure.
- Responses to cGMP are stringently compartmentalized in functionally relevant subcellular microdomains by cGMP-hydrolyzing enzymes phosphodiesterases (PDEs), especially by PDE2 and PDE5 families.
- Because adult cardiac myocytes have low cGMP levels, visualization may require highly sensitive biosensors and imaging techniques.

What New Information Does This Article Contribute?

- We developed a novel transgenic mouse that expresses the highly sensitive cytosolic Förster resonance energy transfer (FRET)-based cGMP biosensor red cGES-DE5 in adult myocardium.
- FRET measurements in adult cardiomyocytes reveal strong cGMP-stimulating effect of the C-type natriuretic peptide (CNP) and low basal cGMP levels, which are controlled by NO-sensitive guanylyl cyclase and by the hydrolytic activity of PDE3, with much lower contribution of PDE2 and PDE5.

Because cGMP levels are difficult to measure in cardiac myocytes, we sought to directly monitor real-time cGMP dynamics in adult mouse cardiac myocytes under normal and pathological conditions. This was achieved by the generation of a new transgenic mouse model with cardiomyocyte-specific expression of the red cGES-DE5 biosensor, which measures cGMP with nanomolar sensitivity in a more intact physiological system. The results of such live cell imaging suggest important contributions of therapeutically relevant PDEs and reveal the previously underappreciated role of PDE3 in the regulation of cGMP levels. These findings, and our observation that PDE5 is upregulated in a mild cardiac hypertrophy model, might serve as a basis for new, more precise therapeutic approaches to the treatment of cardiac disease. In addition, we could observe an exceptionally strong cGMP-stimulating potential of CNP, which was less well studied before. This paracrine peptide hormone and its signaling pathway can be further studied as another way to modulate intracellular cGMP and augment protective mechanisms in the failing heart.

**Transgenic Mice for Real-Time Visualization of cGMP in Intact Adult
Cardiomyocytes**

Götz. Cardiomyocyte cGMP-FRET mouse

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Supplemental Methods

Chemicals. Mouse/rat ANP was from Bachem Holding AG (Bubendorf, Switzerland), human/porcine CNP was from Merck (Darmstadt, Germany). BAY-58-2667 was from Adipogen International. BAY-60-7550 was from Santa Cruz (CA, USA). PROLI NONOate (PROLI-NO) was from Cayman (MI, USA). S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP), Diethylamine NONOate (DEA/NO), 8-methoxymethyl-3-isobutyl-1-methylxanthine (8-MMX), cilostamide, 3-isobutyl-1-methylxanthine (IBMX), melatonin, and all other chemicals were from Sigma-Aldrich (Deisenhofen, Germany).

Genotyping of transgenic mice. The founder mice and all heterozygote offsprings used for cell isolations and FRET measurements were genotyped by a standard PCR using the primers : TGACAGACAGATCCCTCCTAT and GGATGCTCAGGTAGTGGTTGT, resulting in a ~690 b.p. fragment on a gel.

Histology, Morphometric analysis and Echocardiography were performed exactly as previously described.¹ For histology and morphometric analysis, wildtype and transgenic hearts were perfused with phosphate buffered saline until blood free and fixed with 4 % Roti Histofix (Roth) at 4°C overnight. One day later, the fixed hearts were embedded in paraffin, and 5 µm cross sections were obtained using a microtom (Leica RM 2165). For further analysis, paraffin cross-sections were dewaxed and rehydrated in xylol (20 min twice) followed by 4 series of decreasing ethanol concentrations (5 min each step). Hematoxylin-eosin stainings were performed at the Department of Pathology, University of Göttingen Medical Center using a standard automated procedure. Briefly, cell nuclei were stained thrice with hematoxylin (2 min each), and then the cytosol was stained twice using eosin (2 min each). Rehydrated cross-sections were mounted and analyzed using a Stemi 2000-C microscope binocular with associated AxioCam ICc1 and AxioVision software (Carl Zeiss MicroImaging, Jena, Germany). For cardiomyocyte dimension analysis, transverse heart sections were incubated with Wheat Germ Agglutinin (WGA, 75 µg/mL) for 30 min in the dark, washed thrice for 5 min with phosphate buffered saline, mounted and observed under Axiovert 200 microscope (Carl Zeiss MicroImaging, Jena, Germany). Images were acquired using AxioVision software (Carl Zeiss MicroImaging) and analyzed with ImageJ. The cell diameter was measured in 100 cells from 5 sections per heart.

Cardiomyocyte isolation. Mice were sacrificed under isoflurane anesthesia by cervical dislocation, and the hearts were quickly explanted and transferred into a chamber with ice-cold phosphate-buffered saline. Aorta was cannulated onto a 21G cannula and Langendorff perfused at 37°C and 3 mL/min with Ca²⁺-free perfusion buffer (in mmol/L: NaCl 113, KCl 4.7, KH₂PO₄ 0.6, Na₂HPO₄·2H₂O 0.6, MgSO₄·7H₂O 1.2, NaHCO₃ 12, KHCO₃ 10, HEPES 10, taurine 30; 2,3-butanedione-monoxime 10, glucose 5.5, pH 7.4) for 3 min followed by 30 mL digestion buffer (perfusion buffer containing Liberase DH (0.021 - 0.042 mg/ml, Roche), trypsin (0.025 %, Gibco), and CaCl₂ 12.5 µmol/L).² Thereafter, the atria were cut away, and the ventricles were minced for 30 sec in 2.5 mL digestion buffer. Next, 2.5 mL stopping buffer I (perfusion buffer containing 1% bovine serum albumin (BSA, Sigma) and 50 µmol/L CaCl₂) were added to the cell suspension to stop the digestion. The suspension was homogenized for 3 min using a 1 mL syringe without a needle. After 10 min of sedimentation in a 15 mL Falcon tube, the cardiomyocyte pellet was transferred into the stopping buffer II (perfusion buffer containing 0.5% BSA and 37.5 µmol/L CaCl₂) for subsequent stepwise recalcification up to 1 mmol/L CaCl₂. Cardiomyocytes were plated onto round laminin (Sigma) coated coverslips (24 mm, Thermo Scientific) and incubated at 37°C and 5% CO₂ for 30 - 60 min, before cardiomyocyte culture medium was added. It contained MEM α medium (Life Technologies #51200-087) supplemented with L-glutamine, 1 % BSA, Insulin-Transferrin-Selenium supplement, 10 mmol/L 2,3-butanedione-monoxime, and penicillin/streptomycin. Cells were incubated in the medium for additional 3 hours prior to FRET measurements.

Neonatal rat ventricular myocytes were isolated as described³ and transfected for 24 h with the red cGES-DE5 plasmid (kind gift from Dr. Y. Niino) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol.

Confocal microscopy was performed using Zeiss LSM 710 microscope (Carl Zeiss MicroImaging) equipped with a Plan-Apochromat x63/1.40 oil-immersion objective. Images were acquired for T-Sapphire (405 nm diode laser excitation, 490-520 nm emission range) and Dimer2 (561 nm diode laser excitation, 580-630 nm emission range) and analyzed using ZEN 2010 software (Zeiss).

FRET analysis. FRET measurements in single cardiomyocytes were done at room temperature in a buffer containing in mmol/L: NaCl 144, KCl 5.4, MgCl₂ 1, CaCl₂ 1, HEPES 10, pH=7.4. Offline analysis of the FRET imaging data was performed using Origin 8.5 software. Single GFP and RFP intensities from each experiment were exported from the VisiView software into Excel and Origin to calculate the corrected FRET ratio as (RFP - 0.94x GFP) / GFP, where 0.94 is the donor-acceptor bleedthrough coefficient measured in cells expressing the donor fluorescent protein alone. If not corrected for bleedthrough, small responses, such as to PDE inhibitors show no opposing changes in raw single intensity channels, whereas strong responses, for instance upon stimulation with 100 nmol/L CNP reveal clear opposing changes even in uncorrected single channels.

Working heart experiments. Isolated working hearts from wildtype FVB/NRj mice were perfused with Krebs–Henseleit (KH) buffer at 37°C through the pulmonary vein and left atrium in an anterograde, fluid-ejecting mode.⁴ Fluid ejected from an aortic cannula against a hydrostatic fluid column set at a height to yield a mean aortic pressure (afterload) of 50 mmHg. Venous return (preload) was adjusted to 5 mL/min. After a 20 min equilibration period, the test agents were infused continuously for 10 min via the coronary arteries. Thereafter, left ventricles were dissected and shock-frozen in liquid nitrogen. Tissue cGMP was extracted with ice-cold ethanol (70 %, v/v), measured by radioimmunoassay and normalized to protein contents.⁴ KH buffer contained in mmol/L: NaCl 118, KCl 4.7, MgSO₄ CaCl₂ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, and glucose 11. KH buffer was oxygenated by gassing the solution with 95 % O₂ / 5 % CO₂.

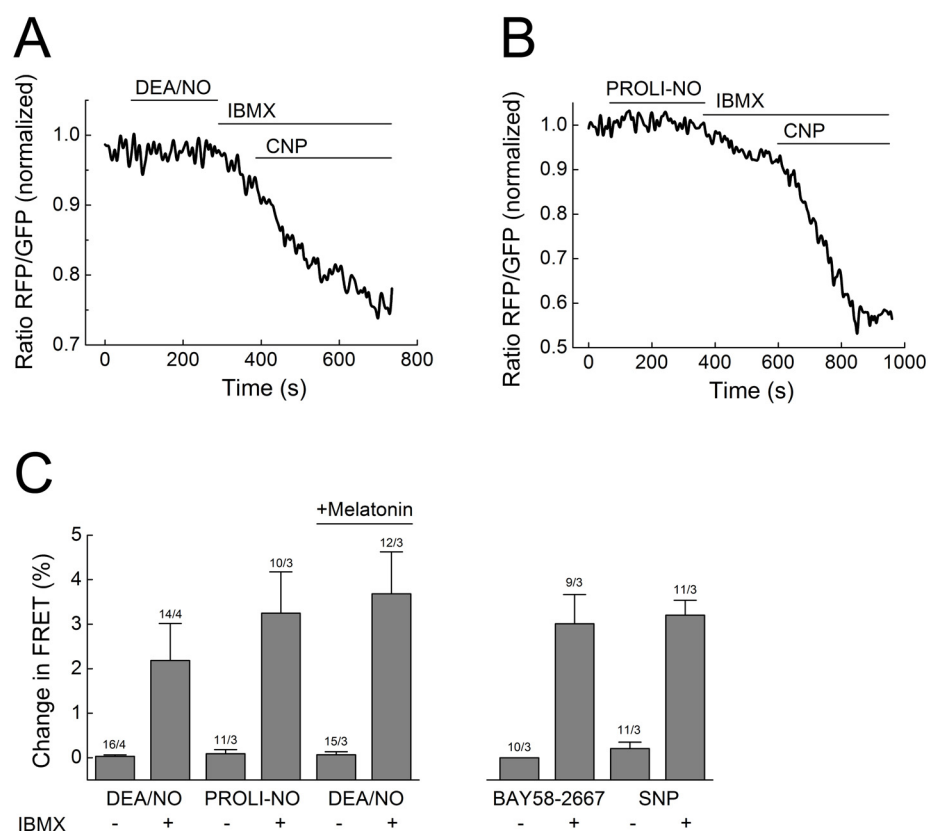
cGMP radioimmunoassay. Freshly isolated adult cardiomyocytes from wildtype or transgenic mice were resuspended in IBMX-containing (500 µmol/L) Tyrode's solution. After 15 min the samples were stimulated for 10 min with 100 nmol/L ANP, 100 nmol/L CNP or 100 µmol/L SNAP, lysed with ethanol and processed for radioimmunoassay analysis as previously described.^{5, 6}

PDE activity assay. Freshly isolated cardiomyocytes were lysed and processed for *in vitro* measurement of cGMP-PDE hydrolyzing activity following the standard method by Thompson and Appleman⁷ in presence of 1 µmol/L cGMP as a substrate, as previously described.⁸ Briefly, freshly isolated cardiomyocytes were collected in stopping buffer I and washed with wash buffer (40 mmol/L Tris, pH 8.0). Thereafter, cells were lysed in 500 µL homogenization buffer (wash buffer containing 10 mmol/L MgCl₂, 1 % Triton X, plus phosphatase- and protease- inhibitors (Roche)). Protein quantification in the lysates was performed using the BCA Protein Assay Kit (Pierce). 75 µg protein were diluted to a total volume of 200 µL homogenization buffer containing PDE inhibitors. Contributions of individual PDE families were calculated from the effects of 10 µmol/L 8-MMX (PDE1), 100 nmol/L BAY-60-7550 (PDE2), 10 µmol/L cilostamide (PDE3), 100 nmol/L tadalafil (PDE5), and 100 µmol/L IBMX (unselective inhibitor). To start the hydrolysis, 200 µL of reaction buffer (wash buffer containing: 10 mmol/L MgCl₂, 10 mmol/L 2-mercaptoethanol, 2 µmol/L cGMP, 1.5 mg/mL BSA, and 0.1 µCi [³H]-cGMP (American Radiolabeled Chemicals Inc.) were added to each sample and incubated at 33°C for 10 min. Next, 200 µL of the stop solution

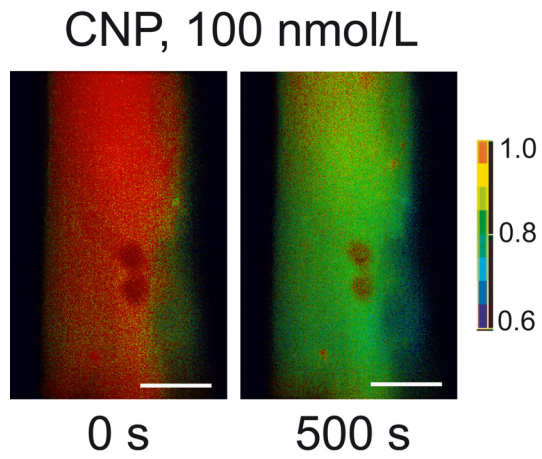
(wash buffer containing 15 mmol/L EDTA, pH 8.0) were added to each sample, followed by heat inactivation at 95°C for 1 min. To hydrolyze the 5'-GMP, samples were incubated with 50 µg of *Crotalus atrox* snake venom (Sigma) at 33°C for 20 min. Finally, each sample was loaded onto a column containing 50 mg AG1-X8 resin (Biorad) for anion exchange chromatography to separate the guanosine, followed by scintillation counting.

Immunoblot analysis. To compare NO-GC protein expression in adult and neonatal cells, freshly isolated adult cardiomyocytes sedimented in stopping buffer II without BSA or neonatal rat myocytes two days after culture were shock frozen. Cells or left ventricular tissue from working hearts were homogenized in a buffer containing in mmol/L: NaCl 150, EGTA 1, CaCl₂ 2, sucrose 300, HEPES 10, pH 7.4 and supplemented with PhosSTOP, protease inhibitor cocktail (Roche, 1 tablet per 10 mL each), and 1 % Triton-X. Proteins were quantified using BCA Protein Assay (Pierce). For NO-GC blots, 10 and 25 µg of total protein were subjected to 10 % SDS PAGE and immunoblotting using the NO-GC α 1 and NO-GC β 1 rabbit polyclonal antibodies (both antibodies together with the recombinant NO-GC β 1 protein were kindly provided by Stepan Gambaryan and Andreas Friebe, University of Würzburg, antibody dilution 1:2000). Immunoblots were scanned, and band densitometry analysis was performed using ImageJ software. For working hearts lysates, anti-P-Ser16 phospholamban (Badrilla 1:5000), anti-GAPDH (HyTest, 1:80.000), and anti- α -tubulin (Sigma, 1:5000) antibodies were used according to manufacturer's protocols. After P-PLN immunoblotting, the membranes were stripped (by 4 min incubation in water and 8 min treatment with 0.2 M NaOH, followed by 4 min washing in water) and probed again with the total anti-PLN A-1 antibody (Badrilla, 1:2500).

Supplemental Figures

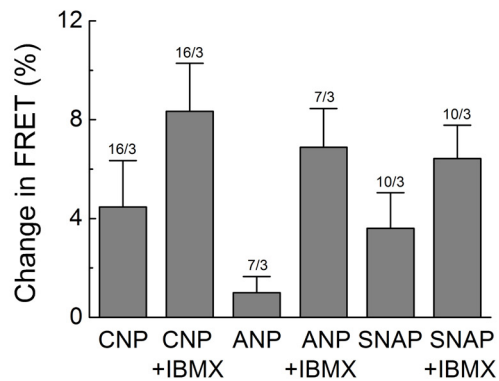


Online Figure I. Effects of rapidly releasing NO-donors, sodium nitroprusside (SNP), NO-GC activator, and an antioxidant on cGMP-FRET signals. Cardiomyocytes were stimulated either with 1 $\mu\text{mol/L}$ DEA/NO (**A**) or with 1 $\mu\text{mol/L}$ PROLI NONOate (PROLI-NO, **B**), and subsequently with IBMX (100 $\mu\text{mol/L}$) followed by CNP (1 $\mu\text{mol/L}$) as a positive control. Both NO-donors did not affect the FRET ratio. Quantification of the data is shown in (**C**). Means $\pm$ SE. SNP, sodium nitroprusside used in the same protocol at 50 $\mu\text{mol/L}$. The numbers of mice and cells per condition are shown above the bars (as n cells/N mice). DEA/NO had no effect, even when cardiomyocyte isolation procedure and measurements were performed in the presence of 100 $\mu\text{mol/L}$ of the reactive oxygen species scavenger melatonin. Likewise, the direct NO-GC activator BAY-58-2667 (cinaciguat, 1 $\mu\text{mol/L}$) did not increase cGMP levels.

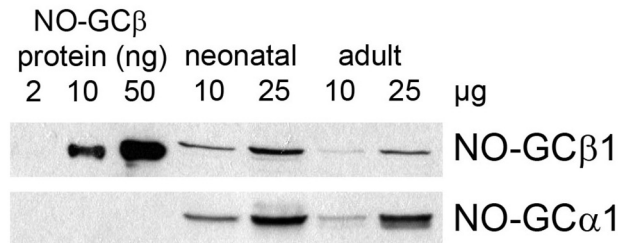


Online Figure II. Representative ratio images of a cardiomyocyte stimulated with CNP. Cell was stimulated with 100 nmol/L CNP for 500 s as described in Figure 2C. RFP/GFP ratio image at baseline (left) and after stimulation (right) is shown. Scale bar, 20 μ m.

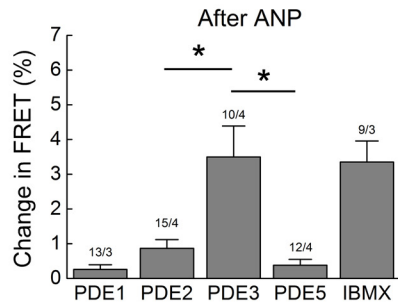
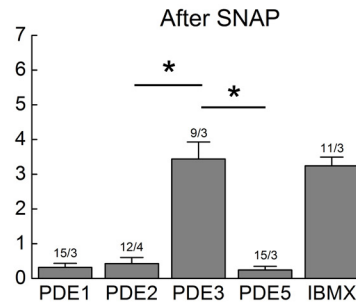
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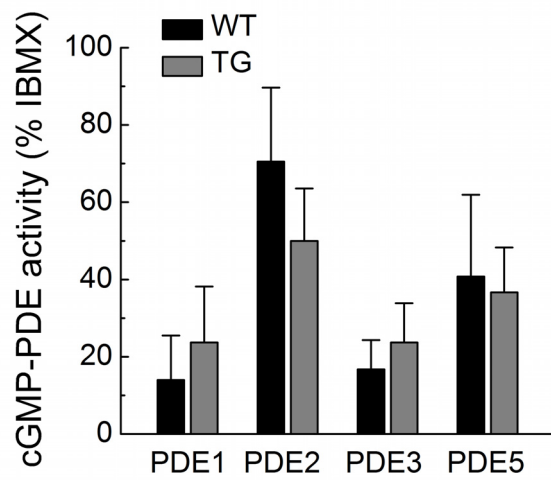
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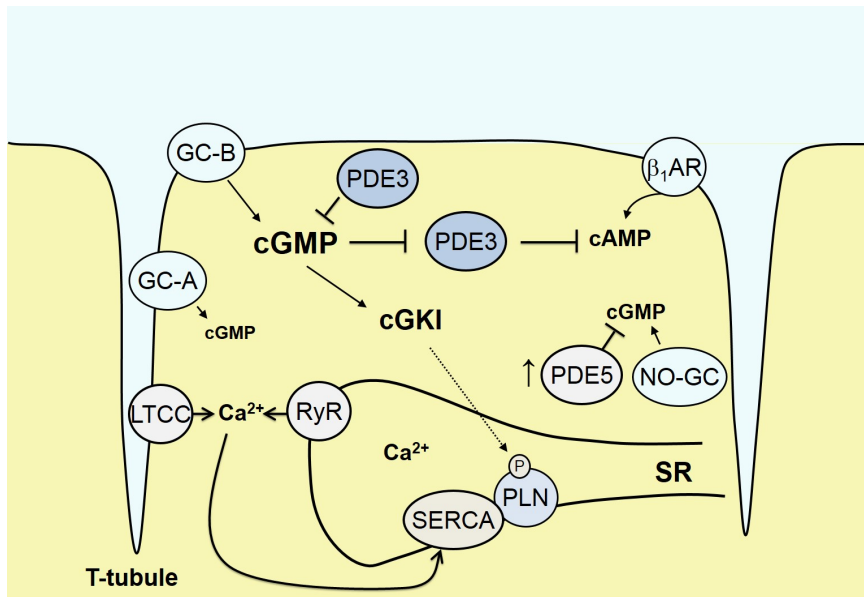
Online Figure III. FRET responses and NO-GC expression levels in neonatal cardiomyocytes. **A**, Changes in FRET in response to 100 nmol/L CNP, 100 nmol/L ANP, 100 μ mol/L SNAP and 100 μ mol/L IBMX in neonatal rat ventricular myocytes transfected with red cGES-DE5 sensor plasmid. Shown are means $\pm$ SE. The number of rat cell isolations and single measured cells analyzed per condition is shown above the bars (as n cells/ N isolations). **B**, Immunoblot analysis of NO-GC levels in neonatal vs. adult cardiomyocytes. 10 and 25 μ g of cell protein were loaded on the gel and probed with NO-GC α or NO-GC β antibodies, also compared to 2, 10 and 50 ng of the recombinant NO-GC β protein. Representative blots (n=3 mouse or rat cell isolations each) are shown. Adult cardiomyocytes express 2.9 ± 0.2 fold less NO-GC than neonatal cells (mean $\pm$ SE, n=3).

A**B**

Online Figure IV. Analysis of PDE inhibitor effects on cGMP levels at prestimulated state. Cardiomyocytes first prestimulated with 100 nmol/L ANP (**A**), or 100 μ mol/L SNAP (**B**), and PDE 1, 2, 3 and 5 inhibitors were applied as described in Figure 4A-E to measure changes in FRET ratio evoked by each PDE inhibitor. Means $\pm$ SE, the numbers of mice and cells per condition are shown above the bars (as n cells/N mice). Significant differences ($p < 0.05$) are indicated with asterisks.



Online Figure V. Contributions of individual PDEs into cGMP hydrolysis measured by *in vitro* assay. Measurement of cGMP-PDE hydrolyzing activity were performed according to the method by Thompson and Appleman⁷ in presence of 1 $\mu\text{mol/L}$ cGMP as a substrate. Shown are means $\pm$ SE, 3-4 mice per group.



Online Figure VI. Schematic diagram showing the proposed PDE isoform regulation of cardiomyocyte cGMP and cAMP levels. GC-B activation by CNP leads to a large increase in cGMP which can inhibit PDE3, thereby increasing cAMP levels. This cGMP also activates cGKI, which in turn phosphorylates phospholamban (PLN). PDE5 activity stringently controls the NO-GC/cGMP pool and is increased after TAC (↑). β₁AR, β₁-adrenergic receptor; LTCC, L-type calcium channel; RyR, ryanodine receptor/calcium release unit; SERCA, sarcoplasmic reticulum calcium ATPase; PLN, phospholamban

Supplemental Tables

Online Table I. Echocardiographic phenotyping of red cGES-DE5 transgenic mice at the age of 6 months. Data are from 8-12 mice per group shown as means $\pm$ SE. LV-ESD, left ventricular end-systolic dimension; LV-EDD, left ventricular end-diastolic dimension; FS, fractional shortening; EF, ejection fraction; PWTD, posterior wall thickness in diastole; PWTS, posterior wall thickness in systole; bpm, beats per minute. None of the parameters was significantly different between the groups at $p=0.05$

Parameter	Wildtype animals	Transgenic animals
LV-ESD (mm)	3.00 $\pm$ 0.11	3.01 $\pm$ 0.08
LV-EDD (mm)	4.20 $\pm$ 0.06	4.21 $\pm$ 0.04
FS (%)	28.8 $\pm$ 2.1	28.5 $\pm$ 1.6
EF (%)	50.1 $\pm$ 3.1	52.1 $\pm$ 2.2
PWTD (mm)	0.76 $\pm$ 0.04	0.74 $\pm$ 0.01
PWTS (mm)	1.09 $\pm$ 0.03	1.12 $\pm$ 0.03
Cardiac output (mL/min)	20.0 $\pm$ 1.3	19.2 $\pm$ 1.3
Stroke volume (μ L)	42.9 $\pm$ 2.2	44.5 $\pm$ 1.3
Heart rate (bpm)	465.3 $\pm$ 14.9	427.8 $\pm$ 20.0

Online Table II. Echocardiographic parameters in transgenic mice 8 weeks after transverse aortic constriction (TAC) or sham surgery. Means $\pm$ SE, n=6 mice per group. * denotes significant differences from the sham group at $p < 0.05$

Parameter	Sham animals	TAC animals
Pressure gradient (mmHg)	4.7 $\pm$ 0.5	84.8 $\pm$ 4.7*
HW/BW ratio (mg/g)	4.34 $\pm$ 0.27	6.58 $\pm$ 0.44*
AWTD (mm)	0.74 $\pm$ 0.04	1.11 $\pm$ 0.05*
PWTD (mm)	0.73 $\pm$ 0.04	1.10 $\pm$ 0.05*
LV-ESD (mm)	2.85 $\pm$ 0.10	3.24 $\pm$ 0.12*
LV-EDD (mm)	3.91 $\pm$ 0.08	4.24 $\pm$ 0.03*
FS (%)	27.22 $\pm$ 1.68	23.7 $\pm$ 2.34
FAS (%)	44.65 $\pm$ 1.49	41.27 $\pm$ 3.08
EF (%)	50.91 $\pm$ 1.43	47.26 $\pm$ 2.40
Heart rate (bpm)	443 $\pm$ 20	483 $\pm$ 24

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Circ Res. 2014;114:1235-1245; originally published online March 5, 2014;

doi: 10.1161/CIRCRESAHA.114.302437

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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