Transgenic Mice for cGMP Imaging

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Rationale: Cyclic GMP (cGMP) is an important intracellular signaling molecule in the cardiovascular system, but its spatiotemporal dynamics in vivo is largely unknown.

Objective: To generate and characterize transgenic mice expressing the fluorescence resonance energy transfer–based ratiometric cGMP sensor, cGMP indicator with an EC$_{50}$ of 500 nmol/L (cGi500), in cardiovascular tissues.

Methods and Results: Mouse lines with smooth muscle–specific or ubiquitous expression of cGi500 were generated by random transgenesis using an SM22α promoter fragment or by targeted integration of a Cre recombinase–activatable expression cassette driven by the cytomegalovirus early enhancer/chicken β-actin/β-globin promoter into the Rosa26 locus, respectively. Primary smooth muscle cells isolated from aorta, bladder, and colon of cGi500 mice showed strong sensor fluorescence. Basal cGMP concentrations were <100 nmol/L, whereas stimulation with cGMP-elevating agents such as 2-(N,N-diethylamino)-diazenolate-2-oxide diethylammonium salt (DEA/NO) or the natriuretic peptides, atrial natriuretic peptide, and C-type natriuretic peptide evoked fluorescence resonance energy transfer changes corresponding to cGMP peak concentrations of ≈3 μmol/L. However, different types of smooth muscle cells had different sensitivities of their cGMP responses to DEA/NO, atrial natriuretic peptide, and C-type natriuretic peptide. Robust nitric oxide–induced cGMP transients with peak concentrations of ≈1 to >3 μmol/L could also be monitored in blood vessels of the isolated retina and in the cremaster microcirculation of anesthetized mice. Moreover, with the use of a dorsal skinfold chamber model and multiphoton fluorescence resonance energy transfer microscopy, nitric oxide–stimulated vascular cGMP signals associated with vasodilation were detected in vivo in an acutely untouched preparation.

Conclusions: These cGi500 transgenic mice permit the visualization of cardiovascular cGMP signals in live cells, tissues, and mice under normal and pathological conditions or during pharmacotherapy with cGMP-elevating drugs. (Circ Res. 2013;113:365-371.)

Key Words: biosensing techniques ■ cyclic GMP ■ fluorescence resonance energy transfer ■ microscopy, fluorescence, multiphoton ■ muscle, smooth ■ vasodilation

The second messenger, cyclic GMP (cGMP), controls cardiovascular, neuronal, and other physiological functions.1,4 It is generated from GTP by guanylyl cyclases, which are stimulated by various signaling molecules and hormones such as nitric oxide (NO) and natriuretic peptides.5 It is degraded to GMP by phosphodiesterases, which are themselves often regulated by cGMP or cyclic AMP.6 cGMP can also be excreted by transport proteins located in the plasma membrane.7 Dysfunctions of the cGMP signaling cascade have been linked to a number of disorders such as retinal degeneration,8 Alzheimer disease,9,10 metabolic syndrome,11 and arterial hypertension.11,12 Drugs that increase the intracellular cGMP concentration are successfully used in the clinics, for instance, organic nitrates for the treatment of angina pectoris or the phosphodiesterase-5 inhibitor sildenafil (Viagra) for erectile dysfunction and pulmonary hypertension. However, the mechanisms that underlie the multiple roles of cGMP in (patho-)physiology and therapy are not fully understood, in part because it is difficult to monitor cGMP signals in live cells or tissues. Protein-based fluorescent biosensors are powerful tools for real-time imaging of cGMP in native cells.13,14 In this study, we have generated and characterized, to the best
Methods

Transgenic cGi500 sensor mice were generated by random or targeted transgenesis in oocytes or embryonic stem cells, respectively. Epifluorescence fluorescence resonance energy transfer (FRET) microscopy was used to monitor intracellular cGi500 in primary cells and tissues isolated from sensor mice and in the cremaster microcirculation of anesthetized sensor mice. cGi500 in blood vessels of the skin was imaged in dorsal skinfold chambers by multiphoton FRET microscopy. For details on mouse generation, cell isolation, cGi500 measurements by FRET microscopy and ELISA, expression analysis by reverse-transcription polymerase chain reaction, and statistics, an expanded Methods section is available in the Online Data Supplement.

Results

Generation of cGi500 Transgenic Mice

To generate transgenic cGi500 sensor mice, we have used the recently developed, improved sensor cGi500 indicator with an EC\textsubscript{50} of 500 nmol/L (cGi500), which displays fast binding kinetics and exquisite selectivity for cGMP over cyclic AMP.\textsuperscript{15} It consists of the tandem cGMP-binding domains of the cGMP-dependent protein kinase type I (white) flanked by cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). The detection of cGMP is based on a decrease of FRET from CFP to YFP on binding of cGMP (Figure 1A). Transgenic cGi500 mice were generated by the following 2 strategies: (1) random integration of an expression cassette driven by a 445-bp fragment of the smooth muscle–specific SM22\textalpha promoter (SM22-cGi500 mice; Online Figure IA) and (2) targeted knock-in into the Rosa26 locus of a Cre recombinase–activatable expression cassette driven by the ubiquitous cytomegalovirus early enhancer/chicken β-actin/rabbit β-globin (CAG) promoter (R26-CAG-cGi500 mice; Online Figure IIB). Note that the R26-CAG-cGi500(L1) mouse line that we describe here carries the permanently activated sensor transgene (designated L1 allele in Online Figure IIA).

Western blot analysis of tissues from SM22-cGi500 mice with an anti–enhanced green fluorescent protein antibody of our knowledge for the first time, transgenic mouse lines that express a cGMP indicator.
demonstrated expression of the sensor in smooth muscle–rich tissues such as aorta, bladder, and colon but not in skeletal muscle or other nonsmooth muscle tissues (Online Figure IB and data not shown). Expression of the sensor in bladder and colon was unexpected because the SM22α-445 promoter fragment that was used in our transgene was originally reported not to be active in visceral smooth muscle. The cGi500 protein detected in lysates of the bladder and colon by Western blotting (Online Figure IB) might have been derived from vascular smooth muscle. However, whole-mount preparations analyzed by fluorescence microscopy confirmed that the sensor was indeed expressed in bladder and colon smooth muscle (Online Figure IC). Differences in the expression pattern of SM22α-445 promoter–driven transgenes may be attributable to different chromosomal integration sites in different transgenic mouse lines. Primary smooth muscle cells isolated from the aortic vasculature, bladder, and colon (vasculature smooth muscle cells [VSMCs], bladder smooth muscle cells [BSMCs], and colon smooth muscle cells [CSMCs], respectively) of SM22-cGi500 mice showed strong fluorescence of the sensor in ≈25% of the cells, indicating robust but mosaic expression of the transgene (Online Figure ID). At the single-cell level, the fluorescence was distributed homogeneously in the cytoplasm without nuclear localization. On the basis of the analysis of sensor fluorescence, the R26-CAG-cGi500(L1) mice showed widespread and uniform cGi500 expression in most organs and tissues of adult mice (Online Figure IIIA), in embryos (Online Figure IIIB), and in primary cells derived from them (Online Figure IIIC and IID and data not shown). The cGi500 transgenic mice were apparently healthy and fertile and had a normal life expectancy. Neither of the 2 mouse lines showed signs of toxicity of sensor expression. The mean arterial blood pressure of SM22-cGi500 mice as measured by the tail cuff method was not significantly different from that of nontransgenic control littermates (84±2 mmHg [n=5] versus 78±5 mmHg [n=4]).

**cGMP Imaging in Primary Smooth Muscle Cells**

To validate the new cGi500 transgenic mice for cGMP imaging, we monitored FRET changes in smooth muscle because it is one of the best-characterized target tissues of cGMP and smooth muscle relaxation is a classic function of cGMP. First, we analyzed primary VSMCs isolated from SM22-cGi500 mice by using an epifluorescence-based FRET imaging setup. The cells were superfused for 2 minutes with C-type natriuretic peptide (CNP) or with the NO-releasing compound 2-(N,N-diethylamino)-diazenolate-2-oxide diethylammonium salt (DEA/NO), which activate guanylyl cyclases at the plasma membrane or in the cytosol, respectively. Both stimuli induced rapid and reversible changes of cGMP concentrations at the plasma membrane or in the cytosol, respectively. Dose-response experiments (Figure 3A) showed that all 3 cell types responded well to NO, but with different sensitivities, in the following order: BSMCs>VSMCs>CSMCs (corresponding EC50 for DEA/NO=73 nmol/L, 106 nmol/L, 147 nmol/L). The molecular basis of their differential sensitivity to NO is not clear but might reflect different equipment of each smooth muscle cell type with guanylyl cyclases and phosphodiesterases. Semi quantitative reverse-transcription polymerase chain reaction analysis indicated that VSMCs, BSMCs, and CSMCs express similar mRNA levels for the α and β subunits of NO-responsive soluble guanylyl cyclase (Online Figure V). Preincubation with the nonspecific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine strongly potentiated NO-induced cGMP signals in all cell types (Figure 3B). The relatively selective phosphodiesterase-5 inhibitor sildenafil had a comparatively
weak effect on all 3 cell types (Figure 3C). We conclude that
cGMP levels in VSMCs, BSMCs, and CSMCs are controlled
by phosphodiesterase-5 and other phosphodiesterases; the latter
might at least in part contribute to the differential sensitivity
of these cell types to NO. Further experiments indicated that
these smooth muscle cell types also respond differentially to
atrial natriuretic peptide (ANP) and CNP (Figure 3D). VSMCs
showed relatively weak, but clearly detectable, cGMP signals
on stimulation with ANP and robust signals on stimulation with
CNP; BSMCs did not respond to ANP but responded to CNP
to a similar extent as VSMCs; CSMCs were not responsive to
ANP or CNP. In line with these results, mRNA for the ANP
receptor guanylyl cyclase-A was detected only in VSMCs, and
mRNA for the CNP receptor guanylyl cyclase-B was detected
in VSMCs and BSMCs (Online Figure V). Surprisingly, mRNA
for guanylyl cyclase-B was also expressed in CSMCs (Online
Figure V), which, however, did not increase cGMP levels on
incubation with CNP (Figure 3D). Elucidation of this discrep-
ancy requires further investigations.

Detection of Vascular cGMP Signals in Isolated Tissues and Living Mice

Next, we analyzed cGMP signals in VSMCs of blood vessels
in acutely isolated retinas of SM22-cGi500 mice. As expected,
these vessels showed mosaic sensor fluorescence (Figure 4A).
Nevertheless, on superfusion of DEA/NO, we recorded pro-
nounced cGMP transients in these vessels (Figure 4B). The
peak concentrations of cGMP were ≈1 µmol/L. To validate
the cGi500 mice in vivo, we performed intravital FRET mi-
croscopy of small arteries in anesthetized mice. These studies
were done with R26-CAG-cGi500(L1) mice, which exhibit
stronger and more homogeneous cGi500 expression in small
vessels than SM22-cGi500 mice. At first, the microcirculation
was studied in the cremaster muscle because these arterioles
are amenable to intravital microscopy and exhibit a functional
NO/cGMP pathway leading to vasodilation.23 FRET imaging
was performed with an epifluorescence setup similar to that
used for the analysis of primary cells and isolated retinas. As
illustrated in Figure 5 and in Online Movie I, robust and re-
versible cGMP signals were detected in arterioles on repeated
superfusion of the tissue with DEA/NO. The cGMP transients
reached peak concentrations of >3 µmol/L. Adenosine, a va-
soactive substance that is thought not to affect cGMP levels,
was applied as a negative control.

As a second in vivo model of vascular cGMP imaging, we
used multiphoton FRET imaging of blood vessels in cGi500
mice carrying a dorsal skinfold chamber.24 To induce cGMP
increases, anesthetized animals received intravenous injections
of DEA/NO. This model allows long-term noninvasive and
more physiological imaging than the cremaster preparation, in
which the tissue is acutely exposed and superfused with DEA/
NO. Sensor fluorescence was clearly detectable in the vascu-
larature of the skin (Figure 6A and 6C). Repeated injection of
100 µL DEA/NO (1 mmol/L) resulted in clear increases of the
FRET ratio signal recorded in the vessel wall, indicating
NO-induced vascular cGMP signals (Figure 6B). The cGMP
concentration began to increase shortly (~5–10 seconds) after
DEA/NO injection (Figure 6 and data not shown). The DEA/
NO-stimulated increases of the FRET ratio were derived from
characteristic changes of the CFP and YFP emissions in oppo-
site directions, and injection of 100 µL vehicle (saline) did not
induce such changes (Figure 6B). Moreover, the profiles of the
cGMP transients correlated with the dose of DEA/NO and with
the extent of vessel relaxation (Figure 6D). The NO-induced
vasodilations are also presented in a time-lapse video (Online
Movie II). Note that the video was recorded in the CFP channel
and thus shows sensor fluorescence but not FRET ratio signals.
Interestingly, multiple injections of the lower dose of DEA/NO
(0.1 mmol/L) resulted in desensitization of both the cGMP and
the vasorelaxation response, whereas the higher dose of DEA/
NO (1 mmol/L) evoked sustained responses that could not be
further increased by another injection of the drug (Figure 6D).

Discussion

In this study, we have generated and characterized transgenic
mice expressing the FRET-based cGMP indicator cGi500.
Our results indicate that the cGi500 transgenic mice permit the visualization of cardiovascular cGMP signals in real time in live cells. Importantly, cGMP sensor mice are useful tools to study vascular cGMP signals associated with vasodilation in vivo. However, these experiments have also limitations. First, the maximal NO-induced changes of the FRET ratio signal that were recorded with the multiphoton microscope were \( \approx 5\% \) (Figure 6) as compared with \( \approx 30\% \) with the epifluorescence setup (eg, Figure 5). However, without calibration of the sensor under the conditions of multiphoton imaging, it is not clear whether the lower ratio changes reflect lower cGMP concentrations or physical differences between the imaging setups. Second, it is difficult to estimate the plasma concentrations of NO that induced cGMP elevations in vivo. The doses of the prodrug DEA/NO that were injected into the tail vein in these experiments seem relatively high (100 µL of a 0.1- or 1-mmol/L solution). However, if we assume that NO is released from 100 µL of a 0.1-mmol/L DEA/NO solution during a period of 5 to 10 seconds (half-life of DEA/NO, 2.1 minutes at pH 7.4 and 37°C; 1.5 moles NO per 1 mole of DEA/NO), then the concentration of free NO that evoked cGMP transients (Figure 6D) was roughly 15 to 30 mmol/L, which is presumably in the range of physiological NO concentrations.

Taken together, our results show that cGMP can be readily detected in smooth muscle cells of SM22-cGi500 and R26-CAG-cGi500(L1) mice, both in vitro and in vivo, opening new experimental routes for studying cGMP signaling in smooth muscle. For instance, primary cells isolated from cGi500 transgenic mice could be used to analyze subcellular cGMP compartments. Because these cells express the cGMP sensor throughout the cytoplasm, global cGMP in the cytosol can be visualized by epifluorescence microscopy, whereas local cGMP pools at the plasma membrane can be imaged by confocal microscopy or total internal reflection fluorescence microscopy. Another exciting application of the cGi500 mice is the characterization of NO- or natriuretic peptide–induced cGMP responses in different vascular beds in vivo. Because of its strong and uniform sensor expression, the R26-CAG-cGi500(L1) mouse line might be better suited for most in vivo applications, but the sparser and specific labeling of

Figure 3. Characterization of cyclic GMP (cGMP) signals in different smooth muscle cell types (vascular smooth muscle cells [VSMCs], bladder smooth muscle cells [BSMCs], or colon smooth muscle cells [CSMCs]) isolated from SM22-cGMP indicator with an EC50 of 500 nmol/L (cGi500) mice. A, 2-(N,N-diethylamino)-diazenolate-2-oxide diethylammonium salt (DEA/NO–cGMP) dose-response experiments; original recordings (left) and estimated ∆R/R peak areas (right) upon stimulation for 2 minutes with increasing concentrations of DEA/NO (in nmol/L). For dose-response curves, data were normalized to the curve maximum. Curves are significantly different between BSMCs, VSMCs, and CSMCs (P<0.01 for all pairwise comparisons) with EC50 values (in nmol/L) of 73±8, 106±7, and 147±1, respectively. B and C, Phosphodiesterase inhibition with 3-isobutyl-1-methylxanthine (IBMX; B) or sildenafil (C) potentiates NO-induced cGMP responses. Cells were stimulated with a subsaturating concentration of DEA/NO deduced from dose-response curves in A (eg, VSMCs, 75 nmol/L; BSMCs, 50 nmol/L; CSMCs, 100 nmol/L) before, during, and after incubation with 300 µmol/L IBMX (B) or 30 µmol/L sildenafil (C). Representative original recordings are shown for BSMCs (left). A summary of NO-induced cGMP signals before, during, and after phosphodiesterase inhibition is shown in the bar diagrams. Peak areas were normalized to the first peak of each experiment. Note that in the case of IBMX treatment, the second peak area does not reflect real cGMP levels because the cGi500 sensor becomes saturated until IBMX is removed. D, Differential cGMP responses of VSMCs, BSMCs, and CSMCs to natriuretic peptides. Cells were superfused for 2 minutes with the indicated concentrations (in nmol/L) of atrial natriuretic peptide (ANP; left) or C-type natriuretic peptide (CNP; right). As a positive control, DEA/NO was applied at the end of each experiment (VSMCs, 500 nmol/L; BSMCs, 100 nmol/L; CSMCs, 500 nmol/L). The bar diagram summarizes the cGMP signals induced by 500 nmol/L ANP or CNP in VSMCs and BSMCs. The ∆R/R peak areas were normalized to the control stimulation with DEA/NO. Only VSMCs responded to ANP. All data are means±SEM (n cells in the experiment as indicated in the respective panel). Representative results from ≥4 experiments with independent cell cultures are shown.
smooth muscle cells in the SM22-cGi500 mouse line allows better imaging of individual cells, which are difficult to delineate in the ubiquitous line. In addition, the widespread sensor expression in R26-CAG-cGi500(L1) mice will be useful for real-time monitoring of cGMP signals in many other tissues and cell types under normal and pathological conditions; these mice might also be a valuable tool to identify target tissues of old and new cGMP-elevating drugs. Note that we have also generated a R26-CAG-cGi500(L2) mouse line that carries a loxP-silenced cGi500 transgene (designated L2 allele in Online Figure II). In the basal state, cells of this mouse line express a red fluorescent protein from the L2 allele, but Cre-mediated recombination converts the L2 into the L1 allele (Online Figure II). Recombined cells carrying the L1 allele express the cGi500 sensor and can be used for cGMP imaging (Online Figure VI). Crossbreeding the R26-CAG-cGi500(L2) mouse line with tissue-specific Cre mice allows cGi500 expression to be selectively switched on only in tissues or cell types of interest. Thus, these new cGi500 transgenic mouse lines, which will be made available to the scientific community, provide multiple imaging options to study the mechanisms of cGMP signaling and to discover new sites of cGMP action in vivo in mammals.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

• The cyclic nucleotide cyclic GMP (cGMP) is an important second messenger that transmits signals from nitric oxide and natriuretic peptides.
• Drugs that increase the intracellular cGMP concentration are used for the treatment of erectile dysfunction and pulmonary hypertension.
• The physiological outcome of cGMP signal transduction may depend on the spatiotemporal profiles of the intracellular cGMP signals and the prevalence of global versus local cGMP pools.

What New Information Does This Article Contribute?

• Transgenic mouse lines were generated that express a cGMP indicator protein that changes its fluorescence upon binding of cGMP so that intracellular cGMP can be monitored and quantified in real time in live cells.

• These mouse models provide new opportunities to study cardiovascular cGMP signaling in vivo.

To improve our understanding of the physiological and pathophysiological functions of cGMP and to monitor its spatiotemporal dynamics in native systems, we generated transgenic mouse lines that express an improved cGMP indicator protein based on fluorescence resonance energy transfer. The new cGMP sensor mice were characterized for their use in cGMP imaging. Proof-of-principle experiments showed that cGMP can be visualized in primary cells and tissues isolated from these mice, and in live animals. In addition, we present new biological findings on differential cGMP signals in different types of smooth muscle cells. Because of the design of the transgene construct, expression of the cGMP indicator can be specifically targeted to any tissue or cell type of interest. We anticipate that the cGMP sensor mouse will become a useful tool for monitoring cGMP levels in vivo.
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SUPPLEMENTAL MATERIAL

Transgenic mice for cGMP imaging

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Methods

Animals
Mice were housed in a conventional mouse facility at 22°C and 50-60% humidity in a 12h/12h light/dark cycle with free access to standard rodent chow and tap water. Adult male and female mice were used for experiments. The cGi500 transgenic mice were heterozygous for the transgene. SM22-cGi500 mice were on a mixed B6D2/C57BL6N or C57BL/6N genetic background, and R26-CAG-cGi500(L1) mice were on a mixed 129Sv/C57BL6N genetic background. Animal procedures were reviewed and approved by the Regierungspräsidium Tübingen and the local authorities in Lübeck and Boston in compliance with the humane care and use of laboratory animals.

Generation of SM22-cGi500 Mice
To generate the plasmid pSM445-cGi500, the CMV promoter in pMGSV1 \(^{1}\) was replaced by a 445-bp fragment of the murine SM22\(\alpha\) promoter (SM445), \(^{2}\) followed by insertion of the cGi500 encoding sequence \(^{3}\) between the rabbit \(\beta\)-globin intron and two polyadenylation signals from the rabbit \(\beta\)-globin gene and the SV40 early region. To generate transgenic mice, the BsrBI-digested 4.9-kb fragment of pSM445-cGi500 carrying the transgene (see also Online Figure IA) was microinjected at a concentration of 2 ng/\(\mu\)L into fertilized B6D2F1 oocytes. Founder mice carrying the transgene were identified by PCR analysis of genomic tail DNA with the primers cGi-fwd (5'-GTCACTCGTGAAGACTCG) and cGi500-rev (5'-TGCTCACCATATATTTTGCC), which amplify a 244-bp product of the transgene. PCR-positive SM22-cGi500 mice were backcrossed to C57BL/6N mice and screened for sensor expression by Western blot analysis of smooth muscle-rich tissues with a rabbit polyclonal antibody against EGFP (ab290, Abcam, Cambridge, MA, USA). GAPDH was used as a loading control (anti-GAPDH; #2118, Cell Signaling, Danvers, MA, USA). Out of 11 analyzed founder lines, one [B6;B6D2-Tg(SM445-cGi500)5Feil] showed strong sensor expression in smooth muscle and was suitable for cGMP imaging experiments.

For immunofluorescence staining of primary smooth muscle cells, cells were fixed for 25 min at 37°C in 4% formaldehyde/4% sucrose. After blocking of non-specific binding with 10% normal goat serum/10% BSA, cells were stained with mouse monoclonal anti-EGFP (sc-81045, Santa Cruz, Santa Cruz, CA, USA) and rabbit polyclonal anti-SM22\(\alpha\) (gift from Mario Gimona) followed by Alexa 488- or Alexa 594-coupled secondary antibodies. Nuclei were stained with 1 \(\mu\)g/\(\mu\)L Hoechst 33258 (Sigma-Aldrich Chemie GmbH, München, Germany).

Generation of R26-CAG-cGi500 Knock-in Mice
To generate the targeting vector pR26-CAG-cGi500(L2), the mG sequence of pRosa26-mT/mG \(^{4}\) (Addgene plasmid 17787) was replaced by the cGi500 encoding sequence. \(^{3}\) Details of plasmid construction are available on request. The targeting strategy was based on the knock-in into the ROSA26 locus of a CAG promoter-driven Cre-activatable cGi500 transgene (see also Online Figure II). Gene targeting was performed as described. \(^{5}\) Briefly, 42 \(\mu\)g of KpnI-linearized pR26-CAG-cGi500(L2) were electroporated into R1 ES cells (genetic background: 129/Sv x 129/Sv-CP), \(^{6}\) and after 8 days of selection with 300 \(\mu\)g/mL G418, 200 clones were isolated and expanded. Three correctly targeted ES cell clones carrying the Cre-activatable “L2 allele” were identified by Southern blot analysis of EcoRV-digested genomic DNA with a 5’ probe that binds to the ROSA26 promoter region. \(^{7}\) Targeted R26-CAG-cGi500(L2) ES cells were transiently transfected with a Cre-expressing plasmid (pIC-Cre) \(^{8}\) to generate ES cells with the ‘excised’ R26-CAG-cGi500(L1) allele. ES cell clones were isolated and subjected to Southern blot analysis as described before as well as to fluorescence microscopy to prove excision of mT and activation of cGi500 expression. Out of 96 clones...
analyzed 32 were positive for the Cre-excised “L1 allele”. R26-CAG-cGi500(L2) and R26-CAG-cGi500(L1) ES cells carrying the silenced but Cre-activatable “L2 allele” and the Cre-activated “L1 allele” of the cGi500 transgene, respectively (see also Online Figure II), were injected into 3.5 dpc C57BL/6N blastocysts to generate chimeric mice. Male chimeras were mated to C57BL/6N females to obtain heterozygous R26-CAG-cGi500(L2) mice [B6;129-Gt(Rosa26)Sor<sup>tm1(Actb-tdTomato; cGi500<sup>Flor</sup>)</sup>] or R26-CAG-cGi500(L1) mice [B6;129-Gt(Rosa26)Sor<sup>tm1.1(Actb-cGi500<sup>Flor</sup>)</sup>] on a mixed 129Sv/C57BL6N genetic background. Mice were further backcrossed to C57BL/6N animals. Germline transmission of the targeted alleles was verified by Southern blot analysis of tail DNA. PCR-based genotyping of ear biopsies was done with primers ROSA10 (5'-CTCTGCTGCTCCTCGTCTTCT), ROSA11 (5'-CGAGGGAGATACACAGCAATA), and ROSA4 (5'-TCAATGGCGGGGGGTGTT).<sup>9</sup> ROSA10 and ROSA11 amplify a 330-bp fragment of the wild-type ROSA26 locus, while ROSA10 and ROSA4 amplify a 250-bp fragment of the R26-CAG-cGi500(L2) or R26-CAG-cGi500(L1) allele (see also Online Figure IIA).

For the analysis of cGi500 fluorescence in organs, mice were anesthetized and sacrificed by cervical dislocation. Organs were isolated in ice-cold PBS and whole-mounts were observed with a fluorescence stereo microscope with EGFP filter set (Discovery, Carl Zeiss Microscopy GmbH, Göttingen, Germany). Then, organs were further processed for sectioning; tissues were fixed in 4% paraformaldehyde in PBS at 4°C overnight, cryoprotected in 30% sucrose at 4°C overnight, embedded in O.C.T. compound (Sakura Finetek Germany GmbH, Staufen, Germany), and frozen in 2-methyl butane at -80°C. 10-µm sections were mounted on SuperFrostPlus slides (Carl Roth GmbH, Karlsruhe, Germany). After drying at room temperature and rehydration in PBS, samples were washed 3 times with PBS-T (0.25% Triton X-100 in PBS), mounted with Shandon ImmuMount (ThermoFisher HealthCare, Houston, TX, USA) and observed under a fluorescence microscope with YFP filter set.

FRET Measurements in Primary Cells and Isolated Tissues

Smooth muscle cells were isolated by enzymatic digestion and FRET imaging of cultured cells and tissues was performed with an epifluorescence-based setup as previously described.<sup>5,10</sup> Briefly, smooth muscle-rich tissues were isolated from 2-5 transgenic mice (5- to 10-week-old) in ice-cold PBS. For VSMCs, surrounding fat and connective tissue were removed from the aorta; for CSMCs, the smooth muscle layer was separated and cleaned from fat and blood vessels; for BSMCs, the inner epithelium and surrounding fat were removed. Then, tissues were cut into 5-mm pieces and incubated at 37°C for 45 min with papain (0.7 mg/mL), followed by 10-15 min with collagenase (1 mg/mL) and hyaluronidase (1 mg/mL); tissues were dissociated by pipetting through a 1000-µL pipette tip. Cells (viability >90% as measured by trypan blue exclusion) were suspended in culture medium (DMEM with 4.5 g/L glucose, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 10% fetal bovine serum) and plated into 12-well plates equipped with glass coverslips (6x10<sup>4</sup> VSMCs, 4x10<sup>4</sup> BSMCs, 3x10<sup>4</sup> CSMCs per well). Cells were grown at 37°C and 5% CO<sub>2</sub>. After 3 days, medium was exchanged. Cells were grown for additional 1-4 days in culture medium before they were serum-starved in culture medium without serum for 24-48 h prior to imaging experiments. These cell cultures contained >90% SM22α-positive cells. Fibroblasts were obtained from adult mouse tail tissue. Tail-tips were skinned and minced and then placed into wells of a 12-well plate containing culture medium and glass coverslips. The cells were grown at 37°C and 5% CO<sub>2</sub> for 7-10 days before analysis.

The imaging setup was composed of an Axiovert 200 inverted microscope with EC Plan Neofluar 10x/0.30 air or 40x/1.30 oil objectives and optional 1.6x Optovar magnification (Carl Zeiss Microscopy GmbH, Göttingen, Germany), a light source with excitation filter switching device (Oligochrome, TILL Photonics GmbH, Gräfelfing, Germany), a DualView beam splitter with 516 nm dichroic mirror and CFP and YFP emission filters (480/30 nm and 535/40 nm) (Photometrics, Tucson, AZ, USA), and a EM-CCD digital camera (Retiga 2000R, Ql imaging, Surrey, BC, Canada). To set up the field of view, cGi500 fluorescence was
observed through an YFP filter set (excitation filter 497/16 nm, 516 nm dichroic mirror, emission filter 535/22 nm). For FRET measurements, a CFP excitation filter (445/20 nm) was used together with a 470 nm dichroic mirror and the beam splitter device. The system was operated by Live Acquisition software (TILL Photonics GmbH) and images were recorded with arivis Browser 2D (arivis GmbH, Berlin, Germany).

For cGMP imaging of primary cells, cells were grown on glass coverslips. The coverslips were mounted into a custom-built superfusion imaging chamber, so that they define the chamber bottom. For cGMP imaging of retinal vessels, an acutely isolated retina was immobilized on a MF membrane filter (Millipore) with a 5x2.5 mm hole; the filter was fixed with silicon grease on a coverslip serving as chamber bottom, and the inner layer of the retina faced towards the chamber bottom. Samples were superfused with imaging buffer (in mM: NaCl 140, KCl 5, MgSO4 1.2, CaCl2 2.5, Glucose 5, HEPES 5, pH 7.4) at room temperature using a pump (Pharmacia P-500, GE Healthcare Europe GmbH, Freiburg, Germany) set to 1 mL/min and two injection valves (Pharmacia V-7, GE Healthcare) with 2-mL and 20-mL sample loops connected in series to apply test compounds (diluted in imaging buffer). The solution in the imaging chamber was continuously removed by aspiration.

cGMP Measurements by ELISA
Primary smooth muscle cells were grown in 6-well plates and serum-starved for 24 h. Cells were kept at room temperature in imaging buffer (2 mL/well) for 10 min. At t=10 min, the buffer was replaced with imaging buffer containing 200 nM DEA/NO for 2 min. At t=12 min, cells were washed once with imaging buffer and incubated in imaging buffer for another 3 min (t=15 min). At t=10 min, 12 min, and 15 min, aliquots of the cells were lysed in ice-cold ethanol (700 µL/well). For each time point, cells from 2 wells were pooled. Cells were scraped from the dish with a blue pipette tip, and lysates were centrifuged for 10 min at 20,000 g and 4°C. The ethanol from the cGMP-containing supernatants was evaporated in a vacuum concentrator. The resulting pellet was dissolved in EIA buffer, and cGMP was determined without acetylation with the Cyclic GMP EIA Kit (Cayman Chemical) according to manufacturer's instructions. To estimate the intracellular cGMP concentration, cell numbers per 6-well were determined from trypsinized culture replicates in a Neubauer chamber, and the cell volume was considered to be 1 pL.11

Expression Analysis by RT-PCR
Total RNA was isolated from serum-starved smooth muscle cells using phenol- and guanidine isothiocyanate-containing PEQGold RNAPure according to manufacturer's instructions. The RNA pellet was dissolved in DEPC-treated water and adjusted to 0.1 µg/µL. Semi-quantitative RT-PCR was performed as described1 with primers for soluble guanylyl cyclase (Gucy1a3 or Gucy1b3 encoding the α1 or β1 subunit, respectively)12 and particulate guanylyl cyclases (Npr1 or Npr2 encoding GC-A or GC-B, respectively)12 and, as internal control, for Gapdh.13

Intravital FRET Measurements in the Cremaster Microcirculation
Preparation of the cremaster muscle of mice for intravital microscopy was performed as previously described.14 The skeletal muscle was carefully removed from selected arterioles to reduce fluorescence signals from non-target tissue. The imaging setup was composed of an upright microscope (Axioskop 2 FS, Carl Zeiss Microscopy GmbH) equipped with a water immersion objective (Achromplan 40×/1.75w) which allowed conventional microscopy to identify and select arterioles for examination that exhibited intact blood flow. FRET measurements were performed using dichroic mirrors and filters as described above, as well as a polychromatic light source (Polychrome V, TILL Photonics) set to 420 nm in this setup. A camera (Andor iXon 885 EM-CCD, Andor Technology, Belfast, Northern Ireland) was...
connected to the beam splitter (DualView, Photometrics) for image recording. The tissue was superfused at a rate of 8 ml/min with warmed (34°C) salt solution (in mM: NaCl 118.4, NaHCO₃ 20, KCl 3.8, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2) gassed with 5% CO₂ and 95% N₂ to yield a pCO₂ of ~40 mmHg and a pH of 7.4. The pO₂ at the surface of the tissue was 20-30 mmHg due to contamination with ambient air. Adenosine (1 mM) was dissolved in water, DEA/NO (1 mM) in NaOH (10 mM) and both were added to the superfusion solution using a roller pump to achieve a dilution of 1:100 and thus a final concentration of 10 µM on the tissue.

Intravital FRET Measurements in the Dorsal Skinfold Chamber

Dorsal skinfold chambers were implanted into adult mice as described. Mice were allowed to recover from surgery for at least 14 days. Anesthetized mice were provided with a tail vein catheter for drug injection and imaged using a custom-built setup for multi-photon FRET microscopy of dorsal skinfold chambers. Fluorescent signal was excited by a Spectra Physics MaiTai Ti:Sapphire laser providing 100 fs pulses at 80 MHz repetition rate and 850 nm wavelength. Beam scanning and image acquisition were performed with an Olympus Fluoview FV300 scanning system interfaced with an Olympus BX51WI upright microscope. The objective was an Olympus XLUMPFL20x W/IR water immersion lens (20x, 0.95 N.A.). The fluorescent signal from CFP and YFP was first separated from the excitation beam by a dichroic mirror (750DCSPXR, Chroma), then separated into CFP and YFP channels by another dichroic mirror (505DCLP, Chroma) and two band pass filters (FF01-483/32-25 and FF01-542/27-25, Semrock) and detected by two separate photomultiplier tubes (HC125-02, Hamamatsu). The resolution of the images was 512 x 512 for a total field of view of 600x600µm. The excitation laser power was 400 mw before the scanner and 36 mw at the sample surface. The separation between two consecutive images in a time series was 5 or 10 s. After baseline acquisition, either 100 µL saline or 100 µL DEA/NO (0.1 mM or 1 mM diluted in saline) were injected within 5-10 s through the catheter.

To evaluate FRET ratio signals in the vessel wall it was necessary to account for dynamic changes in vessel diameter, which happened particularly during NO-induced vasodilation. Therefore, image segmentation according to brightness was performed following the strategy of Zhang and colleagues. Images in the CFP channel were used to define a dynamic binary mask with a threshold that was manually set. Within this mask, all bright structures (e.g., the vessel wall) receive the value “1”, while all dark structures (e.g., the vessel lumen) that fall below the threshold are set to be “not a number” (NaN). Then, this dynamic mask was multiplied with the originally recorded CFP and YFP time-lapse images. In the resulting time-lapse images, regions of interest were defined that were large enough to account for movements of the vessel walls throughout the experiment. Average intensities of a region of interest were calculated without background correction for the evaluation of FRET data (see below). Vessel diameters were estimated from time-lapse images in the CFP channel by measuring the distance between two adjacent vessel walls. FRET ratio signals and vessel diameters were normalized to the baseline period recorded at the beginning of each experiment.

Evaluation of FRET Data

Offline analysis was performed with arivis Browser 2D software (arivis GmbH, Berlin) or ImageJ; for further data evaluation Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and Microcal Origin (OriginLab Corp., Northampton, MA, USA) were used. Background-corrected F₄₈₀ and F₅₃₅ signals were used to calculate the F₄₈₀/F₅₃₅ ratio R. ∆F₄₈₀/F₄₈₀, ∆F₅₃₅/F₅₃₅, and ∆R/R traces were obtained by normalization to the baseline recorded for 2-5 min at the beginning of each experiment. For ∆R/R peak area estimation, the Peak Analyzer Module of Origin was used; single cell traces were corrected for baseline drifts by subtracting a linear baseline, and peak borders were manually defined. For EC₅₀ estimation, a dose-
response function was fitted to the mean peak areas, yielding \( A_{\text{min}}, A_{\text{max}}, \log(\text{EC}_{50}) \) and the Hill constant. For normalization, averaged mean peak areas were divided by \( A_{\text{max}} \), and error bars were calculated by \( \frac{\text{err}(\text{area}) + \text{err}(A_{\text{max}})}{A_{\text{max}}} \).

**Statistics**

To compare DEA/NO dose-response curves, a two-tailed student’s t-test was performed. For PDE inhibitor studies with triple DEA/NO stimulation, a ranked ANOVA with repeated measurements was performed on peak areas using SigmaPlot 11 (Systat Software, Inc., Chicago, IL, USA) and Tukey’s post-hoc test to compare individual DEA/NO stimulations. \( P \) values <0.05 were considered to be significant.

**Supplementary References**


Online Movies

Online Movie I. cGMP imaging in a cremaster arteriole of an anesthetized R26-CAG-cGi500(L1) mouse. This movie shows the FRET ratio signal (reflecting the intracellular cGMP concentration) that was recorded by epifluorescence microscopy during the experiment shown in Figure 5. Upon repeated superfusion of 10 µM DEA/NO (indicated in the top right corner) robust cGMP transients were detected in this vessel.

Online Movie II. NO-induced vasodilation observed by multi-photon microscopy in a dorsal skinfold chamber of an anesthetized R26-CAG-cGi500(L1) mouse. This movie was recorded during the experiment shown in Figure 6C and 6D. Three i.v. injections (100 µL) of 0.1 mM DEA/NO were followed by two injections (100 µL) of 1 mM DEA/NO (indicated in the top right corner). The lower dose of DEA/NO resulted in transient vasorelaxations, whereas the higher dose induced a sustained dilation. Note that the movie shows sensor fluorescence recorded in the CFP channel, but not the FRET ratio signal.
Online Figures

Online Figure I. Generation of SM22-cGi500 mice. **A**, The SM22-cGi500 transgene consists of a 445-bp fragment of the murine SM22α gene promoter (P_{SM445}) followed by a rabbit β-globin intron, the cGi500 coding sequence, and two polyadenylation signals (white boxes) derived from the rabbit β-globin gene and SV40. **B**, Western Blot analysis of tissue extracts (4 µg) from transgenic mice (tg/+ and non-transgenic control animals (+/+) with an antiserum against EGFP to detect cGi500 protein; GAPDH was used as a loading control. **C**, Live YFP fluorescence of the smooth muscle layer of the bladder (left) and colon (right). Scale bars, 100 µm. **D**, Primary VSMCs, BSMCs, and CSMCs from SM22-cGi500 mice were analyzed for live YFP fluorescence (upper panels) or by immunofluorescence staining with antibodies against EGFP (green) and SM22α (red); cell nuclei were stained with Hoechst 33258 (blue). Scale bars, 100 µm.
Online Figure II. Generation of R26-CAG-cGi500 mice. A, Schematic diagram of the gene targeting strategy to insert a Cre-activatable cGi500 construct into the intron between exon 1 and 2 (grey boxes) of the murine ROSA26 locus (+ allele). The targeting construct contained the CAG promoter (P_{CAG}) driving the expression of a loxP-flanked (black arrowheads) membrane-targeted tandem-dimer Tomato (mT, red) and of cGi500 (green) before and after Cre-mediated recombination, respectively. Expression cassettes encoding a FRT-flanked (white arrowheads) neomycin resistance gene (Neo) and diphtheria toxin A (DTA) were used for positive and negative selection of ES cell clones. Integration of the targeting construct via homologous recombination results in a “L2 allele” with two loxP sites, and Cre-mediated excision of mT results in a “L1 allele” with one loxP site. Also indicated are EcoRV restriction sites (EV), the probe and DNA fragments used for Southern blot analysis of ES cell clones as well as the primers (half arrows) used for PCR genotyping of mice. Primers P1, P2, P3 in the figure correspond to primers ROSA10, ROSA11, ROSA4 in the methods section, respectively. B, Southern blot of EcoRV-digested genomic DNA of wild-type (+/+), targeted (+/L2) and Cre-recombined (+/L1) ES cell clones. The expected positions of the DNA fragments derived from the respective alleles are indicated to the left.
**Online Figure III. Global expression of cGi500 in R26-CAG-cGi500(L1) mice.** Sensor fluorescence was detected using an EGFP or YFP filter set in (A) unfixed whole mounts (upper) and fixed cryosections (lower) of various organs from a 6-week-old transgenic mouse, (B) transgenic embryos (12.5 dpc), and (C) primary aortic VSMCs or (D) fibroblasts from adult animals. Stars indicate the positions of control samples from non-transgenic littermates. Scale bars, 100 μm.
Online Figure IV. cGMP FRET in primary VSMCs from R26-CAG-cGi500(L1) mice. Cells were analyzed under similar conditions as primary VSMCs from SM22-cGi500 mice (see Figure 1C). Short stimulations (2 min) with CNP (50 nM) or DEA/NO (100 nM) lead to reversible cGMP increases. Cyan, yellow and black traces indicate CFP emission ($F_{480}$), YFP emission ($F_{535}$) and the CFP/YFP emission ratio ($F_{480}/F_{535}$), respectively. Emission intensities and ratios were normalized to averaged baseline signals and are given as $\Delta F/F$ and $\Delta R/R$, respectively. One representative recording out of four experiments is shown.
Online Figure V. Analysis of guanylyl cyclase expression by semi-quantitative RT-PCR. RNA was extracted from VSMCs, BSMCs, and CSMCs isolated from SM22-cGi500 mice. Reverse transcription was performed with 250 ng total RNA using gene-specific primers. PCR reactions were performed with 1/10 of the cDNA and aliquots were taken every third cycle between cycle 25 and 40. A, PCR products obtained for sGC α1 (cycle 37), sGC β1 (cycle 40), GC-A (cycle 40), GC-B (cycle 40), and Gapdh (cycle 34). B, For densitometric evaluation, band intensities of 3 consecutive aliquots were normalized to Gapdh. Shown are mean values ± s.e.m. of 3 cycles. Note that GC-A mRNA was not quantified, because it was detected only in VSMCs. RT-PCR analysis of GC-A and GC-B was repeated with 500 ng total RNA and random hexamer/polyT primers and similar results were obtained.
Online Figure VI. Validation of cells from R26-CAG-cGi500(L2) mice. VSMCs, fibroblasts, and CSMCs were isolated from R26-CAG-cGi500(L2) mice and then transfected by lipofection with a Cre-expressing plasmid. The detection of green fluorescent cells confirmed that Cre-mediated excision of the mT sequence (encoding a membrane-tagged red fluorescent protein, Online Figure IIA) enables cGi500 expression in all analyzed cell types (left panels). To test the functionality of the cGi500-positive cells, FRET imaging was performed (right panels). Cells were stimulated with CNP or DEA/NO (VSMCs with 50 nM CNP, fibroblasts with 100 nM CNP followed by 100 nM DEA/NO, and CSMCs two times with 100 nM DEA/NO). Data shown are mean ± s.e.m.. The number of analyzed VSMCs, fibroblasts, and CSMCs was 8, 10, and 8, respectively. Scale bars, 100 µm.