Cyclic GMP in Vascular Relaxation
Export Is of Similar Importance as Degradation

Christian Krawutschke, Doris Koesling, Michael Russwurm

**Objective**—In the vascular system, cyclic GMP (cGMP) in smooth muscle cells plays an important role for blood vessel relaxation. Intracellular concentrations of cGMP are thought to be determined by the action of cGMP-generating guanylyl cyclases (sensitive to nitric oxide or natriuretic peptides) and cGMP-degrading phosphodiesterases (PDE1, PDE3, and PDE5). Because functionally relevant cGMP elevations are not accessible to conventional methods, we applied real-time imaging with a FRET-based cGMP indicator to follow nitric oxide– and natriuretic peptide–induced cGMP signals in living smooth muscle cells and analyzed the contribution of the miscellaneous cGMP-generating and cGMP-degrading enzymes.

**Approach and Results**—By comparison of cGMP signals in living smooth muscle cells and vascular relaxation of aortic strips in organ bath experiments, we show for the first time that FRET-based cGMP indicators permit the measurement of functionally relevant cGMP signals. PDE5 was the major cGMP phosphodiesterase responsible for reducing nitric oxide– and natriuretic peptide–induced cGMP signals. In contrast, PDE3—involved in the degradation of lower cGMP concentrations—displayed a preference for natriuretic peptide–stimulated cGMP. Unexpectedly, we found that cGMP is transported out of the cells by the ABC transporter multidrug resistance–associated protein 4 and this export turned out to be of similar importance for intracellular cGMP signals as degradation by PDE5. Functionally, inhibition of cGMP export enhanced vascular relaxation as much as inhibition of PDE5.

**Conclusions**—The findings indicate that cGMP export out of smooth muscle cells is a key player in the regulation of smooth muscle cGMP signals and blood vessel relaxation. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.306133.)

**Key Words:** blood supply ■ cyclic GMP ■ natriuretic peptides ■ nitric oxide ■ vasodilation

The intracellular messenger cyclic GMP (cGMP) plays an important role in the vascular and nervous system. In the vascular system, cGMP lowers blood pressure by regulating vascular tone and endothelial permeability; furthermore, cGMP inhibits platelet aggregation. Vascular tone is determined by the contractile status of smooth muscle cells in the vascular wall. In smooth muscle cells, cGMP generated in response to nitric oxide (NO) or natriuretic peptides (NPs) inhibits smooth muscle contraction and thus causes blood vessel relaxation.

In vascular smooth muscle cells, cGMP is synthesized by 2 types of guanylyl cyclases: NO-sensitive guanylyl cyclases that are activated by NO formed in endothelial cells in response to shear stress, and transmembrane guanylyl cyclases that are activated by NPs. Intracellular cGMP levels are under control of cyclic nucleotide–degrading phosphodiesterases; in smooth muscle cells, PDE1, PDE3, and PDE5 are responsible for degradation of cGMP. The elevation of intracellular cGMP is translated into relaxation mainly by the activation of cGMP-dependent protein kinase that phosphorylates target proteins, such as the myosin phosphatase–targeting subunit and the IP3 receptor–associated cGMP kinase substrate.2-5 In addition to cGMP kinase–mediated effects, cGMP has been proposed to cause smooth muscle relaxation indirectly by elevating cAMP. Enhancement of isoproterenol-stimulated cAMP levels by the NO donor nitroprusside has been attributed to competition between degradation of cGMP and cAMP on the level of PDE3.6

In a previous study, a considerably higher potency of NO to relax aortic rings than to increase intracellular cGMP was observed with half-maximally effective concentrations (EC_{50}) differing by 1.5 orders of magnitude (0.1 versus 3 µmol/L GS-NO7). Hence, the detection of cGMP levels by radioimmunoassays is obviously not sensitive enough to detect the small cGMP elevations sufficient to relax the vessels. FRET-based cGMP indicators have been used to analyze cGMP...
signals in smooth muscle cells. In theory, FRET-based indicators should be ideally suited to detect physiologically relevant cGMP signals because the indicators are constructed from the physiological cGMP effectors. However, the ability of these indicators to detect functionally relevant NO- and NP-induced cGMP signals has not been shown, and the impact of different PDEs on those cGMP signals has not been comprehensively analyzed.

Here, we demonstrate that live cell imaging of primary smooth muscle cells with FRET-based indicators detects physiologically relevant cGMP signals as shown by comparison with relaxation of aortic rings in organ bath experiments. NO-induced cGMP signals were more tightly controlled by PDE5 than by PDE3, whereas NP-induced signals were affected by both PDEs to a similar extent. Under the conditions tested, PDE1 did not affect cGMP signals. Intriguingly, not only degradation of cGMP-determined cGMP signals but also export of cGMP by the transporter MRP4 (multidrug resistance–associated protein 4) was as important as PDE5. Consequently, both cGMP-lowering mechanisms had a comparable impact on the resulting vascular relaxation.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
As 10- to 100-fold higher NO concentrations are required to obtain measurable cGMP signals in radioimmunoassays (or ELISAs) than to induce smooth muscle relaxation, we applied a much more sensitive FRET-based intracellular cGMP indicator to analyze cGMP signals in living smooth muscle cells.

Primary aortic smooth muscle cells were prepared from 3- to 6-week-old C57/BL6 mice of either sex and cultured on coverslips. Five days after preparation, cells were infected with replication-deficient adenoviruses encoding a cGMP indicator to analyze cGMP signals in living smooth muscle cells.

First, we obtained concentration response curves for the slowly releasing NO donor GS-NO by analyzing peak cGMP FRET signals (4 minutes after the addition of GS-NO). GS-NO concentrations as low as 1 nmol/L yielded detectable cGMP elevations, the EC50 for GS-NO stimulation was ≈20 nmol/L, and the maximal response was reached between 100 and 1000 nmol/L GS-NO (Figure 1A). To prove the physiological relevance of these cGMP signals, relaxation of aortic rings was analyzed in organ bath experiments. The EC50 (GS-NO) obtained in these experiments (≈50 nmol/L, Figure 1B) was comparable with the EC50 of GS-NO–induced cGMP elevations (≈20 nmol/L) clearly demonstrating that the FRET-based cGMP measurements are sensitive enough to track physiologically relevant cGMP elevations.

cGMP signals depend on the ratio between cGMP synthesis through guanylyl cyclases and cGMP degradation through phosphodiesterases. Aortic smooth muscle cells express 3 families of cGMP-degrading phosphodiesterases, PDE1, PDE3, and PDE5 (Figure I in the online-only Data Supplement). These phosphodiesterases exhibit substantially different cGMP affinities with PDE3 displaying an at least 10-fold higher affinity (KM ≈0.1 μmol/L cGMP) compared with PDE1 and PDE5 (≈1–6 μmol/L). Because no potent PDE1 inhibitor is commercially available, we used vinpocetine at a concentration of 100 μmol/L for PDE1 inhibition. Because the GS-NO–induced cGMP signals remained unaffected, we conclude that PDE1 has a low impact on cGMP signals under the conditions applied (data not shown). To analyze the contribution of PDE3 and PDE5 to cGMP signals, we inhibited these phosphodiesterases by specific inhibitors (10 μmol/L cilostamide or 1 μmol/L sildenafil for inhibition of

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Figure 1. The cyclic GMP (cGMP) indicator cG-i-6000 detects physiologically relevant cGMP signals in primary smooth muscle cells. A, cGMP signals of primary smooth muscle cells elicited by increasing GS-NO concentrations were measured by live cell microscopy using the FRET-based cGMP indicator cG-i-6000. Shown are changes of emission ratio (CER) 4 minutes after start of GS-NO stimulation calculated as detailed in Methods. Values represent meansSEM of independent measurements (n=10–16 coverslips containing smooth muscle cells from 8 preparations were analyzed per condition, data are from measurements depicted in Figure 2A, black trace). B, Aortic rings precontracted with phenylephrine (1 μmol/L) were relaxed with the indicated GS-NO concentrations. Data represent meansSEM of n=9 independent experiments performed in triplicate or quadruplicates; forces before and after phenylephrine contraction were set to 0% and 100%, respectively.
PDE3 or PDE5, respectively) and obtained GS-NO concentration response curves. To our surprise, inhibition of PDE3 did not affect the GS-NO concentration response curve at all (Figure 2A, blue). In contrast, inhibition of PDE5 increased the cGMP levels elicited by 10 and 100 nmol/L GS-NO and thereby shifted the GS-NO concentration response curve by a factor of 2 with EC\textsubscript{50} values of 20 and 10 nmol/L, respectively (Figure 2A, red).

We then analyzed the effects of PDE inhibitors on the decline of cGMP signals after termination of the GS-NO stimulation (Figure 2E and 2F, from 6 minutes onward). Here, sildenafil almost abrogated the decline of cGMP during the following 9 minutes indicating that no other PDE was able to compensate for the missing PDE5 activity (Figure 2E and 2F, red traces). In contrast, the PDE3 inhibitor cilostamide (Figure 2E and 2F, blue traces) only slowed down the cGMP decline such that the signal 4 minutes after termination of GS-NO stimulation was approximately halfway between the maximal level and the level under control conditions without PDE inhibition (∼25% CER corresponding to ∼6 µmol/L cGMP as inferred from the indicator’s EC\textsubscript{50} determined in vitro, Figure II in the online-only Data Supplement).

Together these data show that PDE3 is the predominant PDE for NO-induced cGMP signals, and PDE3 has a minor impact being evident only after termination of NO stimulation.

In accordance with the predominance of PDE5, the additional inhibition of PDE3 did not further increase NO-induced cGMP signals under PDE5-inhibiting conditions (not shown).

### ANP- and CNP-Induced cGMP Signals

Besides NO-sensitive guanylyl cyclases, aortic smooth muscle cells express 2 other types of guanylyl cyclases: ANP receptor guanylyl cyclase A and CNP receptor guanylyl cyclase B both mediating vasorelaxation.\textsuperscript{14} Because cGMP signals elicited by functionally relevant NP concentrations were inaccessible before, we studied ANP- and CNP-induced cGMP signals in smooth muscle and asked whether these signals are regulated by other PDEs than NO-induced signals.

Maximal ANP-elicited cGMP signals were approximately half as high as those elicited by GS-NO (1 µmol/L), and the EC\textsubscript{50} was ∼10 nmol/L ANP (Figure 3A). Sildenafil increased ANP-elicited cGMP signals to an extent comparable with GS-NO-elicited signals and shifted the EC\textsubscript{50} for ANP to 6 nmol/L. The PDE3 inhibitor cilostamide—which had no effect on the GS-NO concentration response curve—shifted the ANP concentration response curve to the left (EC\textsubscript{50}, ∼5 nmol/L), demonstrating the higher impact of PDE3 on ANP-induced cGMP signals.

Both PDE inhibitors abolished the decline of cGMP after termination of ANP stimulation (Figure 3C–3F). Actually,
a further increase in cGMP was observed in the presence of sildenafil showing that cGMP synthesis was not immediately abolished by stopping the ANP superfusion.

Compared with ANP, 10-fold higher concentrations of the guanylyl cyclase B activator CNP were required to elevate cGMP (EC\textsubscript{50} \approx 100 nmol/L, Figure III A in the online-only Data Supplement), which is in accordance with its lower potency to relax aortic rings in organ bath experiments.\textsuperscript{15} Both PDE inhibitors, sildenafil and cilostamide, increased CNP-stimulated cGMP signals and their effects were slightly higher than on ANP-elicited signals. After termination of CNP stimulation, cGMP signals in the absence of PDE inhibitors declined faster than ANP-stimulated signals (Figure IIID and IIIE in the online-only Data Supplement). The PDE inhibitors displayed effects similar to those on NO-induced signals: sildenafil abolished the cGMP decline and cilostamide slowed it down by a factor of \approx 2 thereby leading to cGMP levels halfway between the levels observed in the presence of sildenafil and in the absence of PDE inhibitors.

In sum, whereas PDE5 controls NO- as well as NP-induced signals, PDE3 has a preference for NP-induced cGMP signals.

**Crosstalk Between cAMP and cGMP**

PDE3 is also termed cGMP-inhibited PDE because cGMP can competitively inhibit cAMP degradation, whereby a cGMP signal can be translated into a cAMP signal. We assessed the crosstalk between cGMP and cAMP in smooth muscle cells. First, we tried to analyze whether NO-stimulated cGMP elevations affect cAMP levels and measured cAMP increases with a FRET-based cAMP sensor (Epac2 camps).\textsuperscript{16} However, this cAMP sensor possesses a cGMP affinity roughly equivalent to the one of the cGMP sensor used in the current study (cGi-6000; EC\textsubscript{50} 6 \mu mol/L) and accordingly responded directly to NO-induced cGMP elevations (not shown).

Then, we analyzed vice versa whether isoproterenol-induced cAMP signals were able to elicit cGMP elevations and found that isoproterenol induced a 10% CER of the cGMP indicator (Figure 4A, black trace). Because the cGMP indicator only shows slight responses to millimolar cAMP concentrations in vitro,\textsuperscript{12} the signal can be attributed to elevated cGMP levels. Because PDE3 inhibition by cilostamide did neither elevate cGMP (Figure 4B) nor affect the isoproterenol-induced cGMP increases (Figure 4A, blue trace) even after preincubation (Figure 4C), a hypothetical competition of cAMP with cGMP degradation by PDE3 cannot account for the cAMP-induced cGMP elevations.

**Export of cGMP**

How do isoproterenol-induced cAMP signals increase cGMP then? Besides phosphodiesterases, active transport of cyclic

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**Figure 3.** Impact of PDE3 and PDE5 on ANP-induced cyclic GMP (cGMP) signals of primary smooth muscle cells. **A**, Primary smooth muscles were stimulated by different ANP concentrations in the absence (black) or presence of 1 \mu mol/L sildenafil (red) or 10 \mu mol/L cilostamide (blue). cGMP was measured by live cell microscopy using the cGMP FRET-indicator cGi-6000. Changes of emission ratio (CER) reached 4 minutes after start of the stimulation were calculated as detailed in Methods. Two-way ANOVA was used to assess whether PDE inhibitors significantly changed the response toward GS-NO (F\textsubscript{2,64}=7.062; P=0.002); P values for comparison of sildenafil or cilostamide vs control are depicted in red or blue, respectively. Numbers of independent experiments are given below. **B**, Pseudocolor-coded cGMP levels of smooth muscle cells before stimulation, during stimulation (4 minutes, 100 nmol/L ANP), and 9 minutes after termination of GS-NO stimulation (from left to right). CER were calculated as detailed in Methods, color-coded as depicted by the calibration bar; a grayscale YFP image of the cells was overlaid to dim regions with lower fluorescence thereby producing a more familiar appearance of the cells. C–F, Shown are time courses of the cGMP signals elicited by 4-minute stimulations (indicated by bars) with 1 nmol/L (C), 10 nmol/L (D), 100 nmol/L (E), or 1000 nmol/L (F) ANP under control conditions (black), in the presence of 1 \mu mol/L sildenafil (red), or 10 \mu mol/L cilostamide (blue) in primary smooth muscle measured by live cell microscopy. Values represent mean±SEM of independent measurements (n=76 coverslips, 3–10 coverslips containing smooth muscle cells from 3–7 preparations were analyzed per condition).
nucleotides across the plasma membrane by MRP has been proposed. Of the 2 cyclic nucleotide-transporting MRPs, MRP4 and MRP5, only MRP4 is expressed in smooth muscle, whereas MRP5 is found in the endothelial layer of blood vessels as has been shown by immunofluorescence, Western blot, and RT-PCR.

The existence of an additional cGMP-lowering mechanism besides PDEs is also in accordance with the minor cGMP decline that we observed in the presence of sildenafil after GS-NO removal (Figure 2D and 2E).

Thus, we analyzed the cGMP export by measuring intracellular and extracellular cGMP levels in radioimmunoassays. In accordance with the proposal of a substantial cGMP export especially under NO-stimulating conditions (1 mmol/L GS-NO), 20 and 180 pmol cGMP per 10⁶ cells were found in the extracellular medium and inside the cells, respectively, after 4 minutes. After 1 hour, extracellular cGMP further increased to 55 pmol, whereas the intracellular cGMP declined to 7 pmol.

MK 571, a leukotriene receptor antagonist, is known to be a potent inhibitor of MRP4. Inhibition of MRP4 by MK 571 diminished the extracellular cGMP (to 3 and 20 pmol after 4 minutes and 1 hour, respectively) and increased intracellular cGMP levels from 180 to 300 pmol after 4 minutes (Figure 4D, left). After 1 hour, intracellular levels remained only marginally elevated by MRP4 inhibition (9 versus 7 pmol) because the no longer exported cGMP was degraded by PDEs (Figure 4D, right). In sum, a substantial amount of cGMP was transported out of the cell. In this experimental setting (radioimmunoassays), high concentrations of GS-NO were required to obtain cGMP signals.
Impact of cGMP Export on Intracellular cGMP Signals

Therefore, we asked whether transport of cGMP affects physiological cGMP signals and analyzed the effect of MRP4 inhibition (10 \(\mu\)mol/L MK 571) on cGMP levels. MRP4 inhibition alone increased the cGMP FRET signal to 5.5% CER after 4 minutes (Figure 4E) and ≤10% CER after 15 minutes (not shown). The slower cGMP increase compared the isoproterenol-induced one may well be caused by the time required for MK571 permeation into the cells. Nevertheless, the data demonstrate the impact of cGMP export under basal conditions. To address the possible competitive inhibition of cGMP export by cAMP, we analyzed the effect MRP4 inhibition (by MK-571) under cAMP-elevating conditions (isoproterenol stimulation) and indeed found that the MRP4 inhibitor did not further increase cGMP when MRP4 was already inhibited by cAMP (Figure 4E). To get another line of evidence, we measured intracellular and extracellular cGMP of smooth muscle cells on isoproterenol stimulation in the absence and presence of MK571 with the radioimmunoassay. However, as may be anticipated from the previous observations in which radioimmunoassays were not sensitive enough to detect low, but functionally relevant cGMP levels, the cGMP concentrations were below the detection limit (not shown).

Then, the effect of MRP4 inhibition on GS-NO concentration response curves was assessed, and MRP4 inhibition was found to markedly increase GS-NO–induced cGMP signals (Figure 5A). At the half-maximally effective GS-NO concentration, MRP4 inhibition potentiated the cGMP signal to nearly maximal levels (40%) and thereby shifted the curve by a factor of ≈2.7. Thus, MRP4 had a comparable impact on NO-induced cGMP signals as PDE5.

After termination of stimulation with a half-maximally effective GS-NO concentration (Figure 5D), the FRET signal rapidly declined to ≈20% in the presence of the MRP4 inhibitor compared with 5% in its absence. This demonstrates that MRP4 is involved in the control of lower cGMP levels (<25% CER). Because cGMP also declined only marginally after termination of maximal GS-NO stimulation in the presence of the MRP4 inhibitor (to 32% CER versus 10% in control; Figure 5E), MRP4 is also involved in the control of higher cGMP levels (>25% CER).

Next, we asked whether both cGMP-lowering mechanisms, cGMP transport and cGMP degradation, independently contribute to the shaping of cGMP signals and added PDE inhibitors (sildenafil and cilostamide) on top of MK571 inhibition at submaximal GS-NO (3 nmol/L). PDE inhibition further increased cGMP signals under MRP4-inhibiting conditions thereby demonstrating cGMP transport and degradation are distinct processes in the control of cGMP signals (Figure 6).

MRP4 inhibition potentiated ANP-stimulated cGMP signals in a comparable manner albeit the decline after termination...
of ANP stimulation was completely abolished by MRP4 inhibition (Figure IV in the online-only Supplemen). Again, the slower decline can at least partially be attributed to the slower deactivation of guanylyl cyclase A—similarly as in the above-mentioned experiments with PDE inhibitors.

The results demonstrate that a substantial amount of cGMP is transported out of the cell and that cGMP export significantly affects intracellular cGMP levels.

**Functional Effect of cGMP Export on Blood Vessel Relaxation**

To evaluate the functional effect of MRP4 on blood vessel tone, NO-induced blood vessel relaxation was measured in organ bath experiments. For comparison, we first analyzed the impact of PDE inhibitors on NO concentration response curves. PDE5 inhibition by sildenafil increased the GS-NO sensitivity by a factor of 2 (Figure 7A). In contrast, PDE3 inhibition by 10 µmol/L cilostamide as used in the cGMP FRET measurements almost completely relaxed aortic rings already in the absence of NO probably by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration. A lower concentration of 0.1 µmol/L cilostamide elicited a 25% relaxation in the absence of GS-NO by increasing the cAMP concentration.

As expected from the cGMP FRET measurements, MRP4 inhibition enhanced relaxation induced by low GS-NO concentrations in aortic rings and thereby shifted the GS-NO concentration response curve by a factor of 2 from ±70 to ±30 nmol/L GS-NO (Figure 7C). The effect of MRP4 inhibition was therefore roughly comparable with that of PDE5 inhibition which demonstrates that active transport of cGMP is a major player within the NO/cGMP cascade.

**Discussion**

Here, we analyzed cGMP signals in primary smooth muscle cells with a FRET-based cGMP indicator. In smooth muscle cells, ≈20 nmol/L of the NO donor GS-NO caused a half-maximal increase of the cGMP FRET signal. It is not possible to calculate the exact amount of NO released from GS-NO because NO release from GS-NO depends on trace amounts of copper ions present in buffer solutions. 

Therefore, we addressed the biological relevance of this GS-NO concentration by analyzing relaxation of aortic rings in organ bath experiments. Here, a similar potency of GS-NO to relax blood vessels was found clearly demonstrating the physiological relevance of the NO concentrations applied and the cGMP signals analyzed.

In our experiments, we used GS-NO as NO donor because its slow NO release makes application in organ bath experiments and FRET measurements far more reliable than the application of DEA-NO. DEA-NO, in contrast, releases NO within minutes and—because of the short half-life of NO—generates short and transient NO peaks. In previous studies analyzing smooth muscle cGMP signals with FRET-based cGMP sensors, DEA-NO was used to elicit cGMP signals. In the more...
recent study, primary vascular smooth muscle cells of mice were analyzed after 7 to 10 days in culture. Different DEA-NO concentrations were applied for 2 minutes, and cGMP signals were analyzed using a cGMP indicator with an EC50 of 500 nmol/L compared with 6000 nmol/L in our study. In this study, 100 nmol/L DEA-NO caused an approximately half-maximal CER and 150 nmol/L already induced the maximal CER compared with an EC50 of ≈20 nmol/L GS-NO determined in our study. The higher concentrations of DEA-NO that release far more NO than GS-NO may well be an indication of DEA-NO’s unreliable application which we tried to avoid using GS-NO.

In the previous study, DEA-NO concentrations of ≈70 nmol/L elicited a half-maximal CER in rat vascular smooth muscle cells. Again, taking into account that the rapid NO release by DEA-NO results in higher NO concentrations, this points to a somewhat lower NO sensitivity of these cells or to DEA-NO’s unreliable application. Moreover, transient cGMP signals were observed in this study not only with DEA-NO but even in the continuous presence of constant NO concentrations elicited by the extremely slow NO donor DETA-NO. This is in contrast to our observations in which even small NO concentrations produced sustained cGMP elevations that did only decline after termination of NO stimulation. The reasons for this discrepancy are unknown but may be related to the use of smooth muscle cells from rat versus mice or to the culture conditions (10–15 days in the presence of 20% bovine growth serum versus 7 days in the presence of 5% FCS in our study).

Besides NO-induced cGMP signals, the FRET indicators permitted the measurement of ANP- and CNP-stimulated cGMP signals. As with GS-NO, concentrations of ANP and CNP that are known to relax blood vessels were sufficient to elicit measurable cGMP signals. According to the higher potency of ANP compared with CNP to relax blood vessels, 10-fold lower concentrations of ANP than of CNP were required to trigger cGMP signals.

PDE5 is presumed to be the major cGMP-degrading PDE in smooth muscle cells. However, the PDEs expressed in smooth muscle cells have different cGMP substrate affinities and PDE5 is also activated by cGMP: (1) directly by cGMP binding to its regulatory domains and (2) indirectly by cGMP-dependent protein kinase–mediated phosphorylation. Thus, the relative contribution of different PDEs to cGMP degradation varies with the cGMP levels reached within a cell and it is important to assess the impact of the respective PDEs at the physiologically relevant cGMP levels. The impact of PDE5 on cGMP signals has been assessed with FRET-based cGMP indicators in the 2 above-mentioned reports, but remained controversial. In 1 study, the PDE5 inhibitor sildenafil abrogated the cGMP degradation after higher DEA-NO concentrations (500 nmol/L). In the other study, sildenafil did not abolish the decline of the cGMP concentration but only increased the area under the curve of the FRET signal by a factor of 2–3.

Here, we analyzed the impact of PDE3 and PDE5 by analyzing 2 parameters, the maximal CER reached by different GS-NO or NP concentrations and the decline of emission ratio after termination of stimulation. At GS-NO concentrations around the EC50, the CER were increased by PDE5 inhibition but not by PDE3 inhibition. In contrast, ANP- or CNP-stimulated cGMP signals near the EC50 ANP or CNP were increased by PDE3 or PDE5 inhibition to a similar extent. Thus, PDE5 preferentially degrades NP-induced versus NO-induced cGMP which is in accordance with the membrane association of PDE5 in smooth muscle.

To address the impact of PDE1, we used vinpocetine. Vinpocetin exerts a rather low potency but is nevertheless the most potent commercially available PDE1 inhibitor. Under the conditions applied, the cGMP signals were not affected.

The cGMP decline after termination of stimulation (by NO or NP) was almost completely abolished by PDE5 inhibition revealing that only PDE5 is able to substantially contribute to cGMP degradation at higher cGMP levels (>6 μmol/L cGMP, equivalent to ≈25% CER). At cGMP levels below ≈6 μmol/L, the contribution of PDE5 to cGMP degradation declined and the impact of PDE3 became apparent.

cGMP has been shown to increase cAMP levels by competing with cAMP degradation through PDE3. We observed vice versa that a cAMP elevation can increase cGMP levels as isoproterenol elicited cGMP FRET signals ≤10% CER. Crosstalk between cAMP and cGMP on the level of PDE3 was ruled out as PDE3 inhibition did not increase cGMP. Because active transport of cAMP as well as cGMP out of smooth muscle cells by MRP4 has been demonstrated, we hypothesized that cAMP increased cGMP by competitively inhibiting the export of cGMP (Figure 8). In support of this notion, transport of ANP-induced cGMP from smooth muscle cells to the extracellular space as well as competitive inhibition of this transport by high intracellular cAMP concentrations has been shown already in 1989. We were able to confirm in radioimmunoassays that a substantial amount of cGMP is transported into the extracellular space. In FRET measurements, inhibition of MRP4 increased intracellular cGMP already under basal conditions (ie, not stimulated by NO or NP) demonstrating that MRP4 is important for basal cGMP homeostasis. In line with the competitive inhibition of cGMP transport by cAMP, MRP4 inhibition did not increase cGMP when MRP4 was already

Figure 8. Regulation of cyclic GMP (cGMP) signals by phosphodiesterases and transport through multidrug resistance–associated protein 4 (MRP4). Cyclic GMP formed by nitric oxide–sensitive guanylyl cyclase (NO–GC) or natriuretic peptide–sensitive, transmembrane guanylyl-cyclase (NP–GC) is either degraded by PDEs (PDE5 or the membrane-associated PDE3) or exported by the transporter MRP4. cAMP, generated by adenyl cyclase (AC) in response to β-adrenergic stimulation, competes with cGMP for the transporter and is thereby able to increase cGMP. For further details, see Discussion.
competitively inhibited by isoproterenol-induced cAMP. Even more impressively, MRP4 inhibition also increased the cGMP signals induced by half-maximal GS-NO or ANP showing that export of cGMP is an important player in the regulation of low micromolar cGMP levels. PDE inhibitors further increased cGMP even when MRP4 was inhibited demonstrating that cGMP export by MRP4 and cGMP degradation are independent contributors for the shaping of cGMP signals.

The functional relevance of MRP4 in the regulation of cGMP levels was assessed in organ bath experiments. As expected from the cGMP FRET measurements, MRP4 inhibition increased the relaxation elicited by submaximal GS-NO concentrations clearly demonstrating that export of cGMP through MRP4 has an impact on cGMP-mediated vascular relaxation. In addition to cGMP degradation by PDE5 and PDE3, export of cGMP through MRP4 has a substantial impact on the regulation of intracellular cGMP levels. Whether the crosstalk between cAMP and cGMP signals on the level of MRP4-mediated export is restricted to smooth muscle or represents a general principle relevant within physiological responses awaits further investigation.

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Disclosures

None.

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Significance

The intracellular signaling molecule cyclic GMP (cGMP) plays a key role in vascular relaxation. Several classes of drugs are targeted to increase cGMP: the nitrovasodilators used in the treatment of angina pectoris, the phosphodiesterase 5 inhibitors used in erectile dysfunction and pulmonary hypertension, and the new ciguat guanylyl cyclase activators likewise approved for pulmonary hypertension. Despite the well-established role of cGMP in vascular relaxation, the underlying intracellular cGMP signals were barely measurable to date. Using a new real-time imaging method, we visualized those functionally relevant cGMP signals in smooth muscle cells and found an important impact of cGMP transport out of the cells by a member of multidrug resistance-associated family of proteins (MRP4). Because inhibition of the transporter enhanced vascular relaxation as much as inhibition of phosphodiesterase 5, cGMP transport out of smooth muscle cells emerges as a new, important player in blood vessel relaxation and possibly as a new pharmacological target.
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SUPPLEMENTAL MATERIAL

Detailed Methods

Preparation of primary smooth muscle cells from murine aorta

Aortae of 2 mice (3 to 6 weeks old C57/BL6) were rinsed with buffer A (in mM: L-glutamate 85, NaCl 60, KCl 5.6, MgCl₂ 2, HEPES 10, pH 7.4), stripped of connective tissue, cut into pieces of 2-5 mm length and digested (40 min, 37 °C; papain 0.7 g/l, DTT 1g/l, BSA 1g/l in 1ml buffer A). After centrifugation (8 min, 200 g), the supernatant was discarded and the aortic pieces were further digested (20 min, 37 °C, collagenase 1 g/l, hyaluronidase, 1g/l, BSA 1g/l in 1ml buffer A; resuspended after 10, 15 and 20 min 10 times with a 1,000 µl pipet). Subsequently, 10 ml of culture medium (DMEM with 4.5 g/l glucose, 1 mM pyruvate, 4 mM glutamine, 5% FCS, 1% penicillin/streptomycin) were added, debris was removed using a 100 µm cell strainer and cells were collected by centrifugation (8 min, 200 g). Cells were seeded on coverslips at a density of 10⁵ cells/cm² for live cell imaging or at 3 x 10⁴ cells/cm² for radioimmunoassay and maintained in culture medium in a humidified 5% CO₂ atmosphere at 37 °C. Identity of the cells was checked by immunofluorescence staining against smooth muscle alpha actin which labelled >95% of the cells.

Expression of the cGMP indicator

Replication-defective adenoviral vectors with an insert coding for cGi-60001 were created by the AdEasy method following a Nature protocol with modifications: Subsequent to steps 1-22, steps 21-22 were repeated until the titer was sufficient (determined as in step 25) and viral supernatants were stored without CsCl purification at -20 °C. For expression of the indicator in smooth muscle cells on coverslips, the medium was exchanged by culture medium without FCS and viral supernatant was added at a multiplicity of infection of 1,000. Three hours later, 5% FCS were added and cells were maintained for two additional days before imaging.

Live cell imaging of intracellular cGMP

Imaging was performed as described in. In short, coverslips with smooth muscle cells mounted on an inverted fluorescence microscope (Zeiss Axiovert 200 with a 10x objective) were superfused with PBS. Fluorescence of the indicator was excited at 431 nm using a polychrome V (Till Photonics, Munich) for 200-600 ms (depending on fluorescence intensity) every 2s. The resulting CFP and YFP emissions were simultaneously recorded using a beam splitter (Optical Insights, equipped with a 505 nm dichroic mirror and 465/30 nm and 535/30 nm bandpass filters) and a CCD camera (Sensicam QE, pco imaging). Images were analysed using ImageJ. In short, a background image (obtained without cells) was subtracted and the CFP emission images were pixel-wise divided by the YFP emission images (such that a cGMP increase is reflected by a ratio increase). Per coverslip, 15-40 cells were selected as regions of interest (ROI). To calculate the depicted change of emission ratio (% CER), the ratios within each ROI were normalized to the corresponding baseline ratio recorded within the first 1.5 min of the measurement. Usually, the mean CER of the selected ROIs was similar to the CER of the whole viewing field (calculated for all pixels that were brighter than >5-10% of the intensity of the brightest pixels in the baseline images). After 2 min, stimulation was performed by addition of the indicated drugs (GS-NO or ANP and PDE inhibitors or MK 571 as indicated) to the superfusion solution; after 4 min of stimulation, GS-NO or ANP stimulation was terminated and only PDE inhibitors or MK 571 in PBS or PBS alone were superfused as indicated.
Isometric measurement of aortic ring relaxation

Eight murine aortic rings (1-1.5 mm, intact endothelium) mounted on myographs (700 MO, Danish Myo Technology, equipped with a PowerLab 8/30 AD converter, AD Instruments) in 5% CO\textsubscript{2}/95% O\textsubscript{2}-gassed Krebs-Henseleit solution (in mM: NaCl 118, KCl 4.7, CaCl\textsubscript{2} 2.55, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 1.1, NaHCO\textsubscript{3} 25, Glucose 8.3 with diclofenac 3 and L-NAME 1) were adjusted to a pretension of 5 mN. After obtaining a baseline for 20-30 min, rings were precontracted with 1 mM phenylephrine. After development of a stable plateau (40-60 min), PDE or MRP4 inhibitors were applied as indicated and subsequently relaxation was induced with the indicated concentrations of GS-NO. For analysis, forces before and after phenylephrine were set to 0 and 100% contraction, respectively.

Measurement of extracellular and intracellular cGMP in radioimmunoassays

For analysis of cGMP export, 7 days old smooth muscle cells prepared as described above and cultured in 24 well plates (1.9 cm\textsuperscript{2} growth area) were washed twice with PBS and allowed to equilibrate in PBS with 5.6 mM glucose (10 min, 37 °C). After preincubation with or without MK 571 as indicated (10 min, 37 °C), cells were stimulated with GS-NO (1 mM) for 4 or 60 min (37 °C) or with isoproterenol (1 µM). Subsequently, the supernatant of the cells was saved and the cells were lysed with 500 µl ice-cold 70% ethanol. Cells were scraped and debris was removed by centrifugation (20 min, 4 °C, 20,000 g). The supernatant of the cells and the lysed cells were dried under N\textsubscript{2} at 100 °C and cGMP was determined by radioimmunoassay\textsuperscript{5}.

Statistics

Concentration response curves measured with the FRET-based cGMP indicator were statistically analyzed by two-way independent ANOVA in SPSS 22. Normality of the residuals was checked in bar graphs. Concentration response curves obtained in aortic ring relaxation experiments were statistically analyzed using mixed linear models with PDE or MRP4 inhibitor and GSNO concentration as fixed and aortic preparation (i.e. animal) as random effects.

To compare cGMP values of isolated smooth muscle cells measured in radioimmunoassays, all values determined for a given preparation of cells were normalized to the mean cGMP value in the presence of GS-NO alone of that preparation under the respective condition (intra-/extracellular, 4/60 min) to account for differences between preparations and analyzed using a Mann-Whitney U test in SPSS.

References

Suppl. Fig. I PDE activities of smooth muscle cells

PDE activity of smooth muscle cell homogenates (after 7 days of culture) was determined under control conditions and in the presence of the indicated PDE inhibitor(s) (0.1 µM sildenafil, 1 µM cilostamide or 50 µM vinpocetine) at a substrate concentration of 1 µM cGMP using 3 µg cellular protein as described previously (Jäger et al., 2010, Br J Pharmacol. 161:1645-60). (A) Shown are means ± SEM if independent measurements performed with \( n=4 \) or 8 preparations (given by the numbers) in duplicates. (B) Shown are dot plots of PDE activities depicted in panel A expressed as percent inhibition compared to the control value obtained with the same preparation.

Data in panel A were analysed by Related-Samples Friedman’s Two-way ANOVA by Ranks (\( p=0.004 \)). Inhibition by Sil, Cil or Vin is significantly different from control as tested by one-sided Wilcoxon signed rank test in SPSS (\( p=0.004 \) for each pairwise comparison) corrected by the Holm-Bonferroni method for multiple testing. Inhibition by the combination of Sil, Cil and Vin is statistically not significantly different from control as the Wilcoxon signed rank test has no power to detect differences with \( n=4 \) measurements.
Suppl. Fig. II Calibration of the cGMP indicator cGi-6000 with cGMP and cAMP

The response of the recombinantly expressed cGMP indicator cGi-6000 to known cGMP and cAMP concentrations was determined in broken cell preparations in a spectrofluorimeter. Fluorescence was excited at 436 nm, CFP and YFP emissions were continuously recorded at 475 and 525 nm, respectively, and the emission ratio at 475 to 525 nm was calculated. After addition of the indicated concentrations of cGMP or cAMP, the changes of emission ratio (CER) were obtained by normalizing to the initial emission ratio determined in the absence of cGMP. Results are means ± S.D. of ≥ three measurements.

Suppl. Fig. III  Impact of PDE3 and PDE5 on CNP-induced cGMP signals of primary smooth muscle cells

(A) Primary smooth muscle were stimulated by different CNP concentrations in the absence (black) or presence of 1 µM sildenafil (red) or 10 µM cilostamide (blue). cGMP was measured by live cell microscopy using the cGMP FRET-indicator cGi-6000. Changes of emission ratio (CER) reached 4 min after start of the stimulation were calculated as detailed in Methods. Two-way ANOVA was used to assess whether PDE inhibitors significantly changed the response towards GS-NO ($F_{2, 80} = 13.51$, $p=9 \times 10^{-6}$); p values for comparison of sildenafil or cilostamide vs. control are depicted in red or blue, respectively. Numbers of independent experiments are given below.

(B-E) Shown are time courses of the cGMP signals elicited by 4 min stimulations (indicated by bars) with 1 nM (B), 10 nM (C), 100 nM (D) or 1000 nM (E) CNP under control conditions (black), in the presence of 1 µM sildenafil (red) or 10 µM cilostamide (blue) in primary smooth muscle measured by live cell microscopy.

Values represent means ± S.E.M. of independent measurements (n=92 coverslips, 5-12 coverslips containing smooth muscle cells from 3-7 preparations were analysed per condition).
Suppl. Fig. IV Impact of cGMP export on ANP-induced cGMP signals of primary smooth muscle cells

(A) Primary smooth muscle were stimulated by different ANP concentrations in the absence (black) or presence of 10 µM of the MRP4 inhibitor MK 571 (green). cGMP was measured by live cell microscopy using the cGMP FRET-indicator cGi-6000. Changes of emission ratio (CER) reached 4 min after start of the stimulation were calculated as detailed in Methods. Two-way ANOVA was used to assess whether MK 571 significantly changed the response towards GS-NO ($F_{1, 36} = 32.02, p = 2 \times 10^{-6}$). Numbers of independent experiments are given below.

(B-E) Shown are time courses of the cGMP signals elicited by 4 min stimulations (indicated by bars) with 0.1 nM (B), 1 nM (C), 10 nM (D), 100 nM (E) or 1000 nM (F) ANP under control conditions (black) or in presence of 10 µM MK 571 (green) in primary smooth muscle measured by live cell microscopy.

Values represent means ± S.E.M. of independent measurements (n=46 coverslips, 3-6 coverslips containing smooth muscle cells from 3-5 preparations were analysed per condition).