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Review Article – Themed Issue

Visualising and understanding cGMP signals in the cardiovascular system

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Abbreviations

ANP	atrial natriuretic peptide
BNP	B-type natriuretic peptide
cAMP	cyclic adenosine 3’-5’-monophosphate
CFP	cyan fluorescent protein
cGK	cGMP-dependent protein kinase
cGMP	cyclic guanosine 3’-5’-monophosphate
CNG	cyclic nucleotide-gated
CNP	C-type natriuretic peptide
DEA-NO	diethylamine NONOate
ELISA	enzyme-linked immunosorbent assay
FRET	Förster/fluorescence resonance energy transfer
GC-A	guanylyl cyclase A
GC-B	guanylyl cyclase B
GFP	green fluorescent protein
GSNO	S-nitrosoglutathione
IBMX	3-isobutyl-1-methylxanthine
NO	nitric oxide
NO-GC	NO-sensitive guanylyl cyclase
PDE	phosphodiesterase
SNAP	S-nitroso-N-acetylpenicillamine
VSMC	vascular smooth muscle cell
YFP	yellow fluorescent protein

Abstract

cGMP is an important signalling molecule in humans. Fluorescent cGMP biosensors have emerged as powerful tools for the sensitive analysis of cGMP pathways at the single-cell level. Here, we briefly outline cGMP's multifaceted role in (patho-)physiology and pharmacotherapy, and then summarise what new insights cGMP imaging has provided into endogenous cGMP signalling and drug action, with a focus on the cardiovascular system. Indeed, the use of cGMP biosensors has led to several conceptual advances, such as the discovery of local, intercellular, and mechanosensitive cGMP signals. Importantly, single-cell imaging can provide valuable information about the heterogeneity of cGMP signals *within* and *between* individual cells of an isolated cell population or tissue. We also discuss current challenges and future directions of cGMP imaging, such as the direct visualisation of cGMP microdomains, simultaneous monitoring of cGMP and other signalling molecules, and, ultimately, cGMP imaging in tissues and animals under close-to-native conditions.

Keywords: nitric oxide, natriuretic peptide, guanylyl cyclase, phosphodiesterase, transgenic mice, cardiomyocyte, VSMC, platelet, FRET microscopy, imaging, signalling, compartmentation, microdomain, cell heterogeneity, biosensor

1. Introduction: cGMP in health, disease, and pharmacotherapy

The cyclic nucleotide, cyclic guanosine 3'-5'-monophosphate (cGMP), is increasingly recognised as an important second messenger that controls multiple cellular functions in humans (Beavo & Brunton, 2002; Hofmann, 2019; Kemp-Harper & Feil, 2008). Drugs that increase the intracellular cGMP concentration are among the most successful developments in the pharmaceutical industry and clinical pharmacology in recent years (Evgenov et al., 2006; Feil & Kemp-Harper, 2006; Oettrich et al., 2016). **Figure 1** illustrates the principle organisation of cGMP signalling pathways in mammals, highlighting some classical functions in the cardiovascular system as well as novel aspects, which were recently discovered by real-time cGMP imaging in living cells and will be discussed in this article. Mammals generate cGMP from GTP via NO-sensitive guanylyl cyclases (NO-GCs, also known as 'soluble' guanylyl cyclases, sGCs) (Koesling et al., 2016) and transmembrane guanylyl cyclases (also known as 'particulate' guanylyl cyclases) such as GC-A, GC-B, and GC-C (Kuhn, 2016). GC-A (also known as NPR1 or NPR-A) binds atrial and B-type natriuretic peptide (ANP, BNP), GC-B (also known as NPR2 or NPR-B) binds C-type natriuretic peptide (CNP), and GC-C is stimulated by guanylin and uroguanylin. cGMP is removed from cells by active export via multidrug resistance-associated proteins (*e.g.*, MRP4 and MRP5) (Sager, 2004) or by hydrolysis to GMP via phosphodiesterases (PDEs) (Conti & Beavo, 2007). The effects of cGMP are mediated by its binding to three classes of cGMP effector proteins: cyclic nucleotide-gated cation channels (CNG channels) (Biel & Michalakis, 2009), cGMP-dependent protein kinases (cGKs, also known as PKGs) (Hofmann et al., 2006), and cGMP-regulated PDEs (Francis et al., 2011). While CNG channels play a role mainly in the sensory system, cGKs and PDEs are widely expressed. The principle cGMP effector in the cardiovascular system is the cGK type I. In addition, certain PDEs, particularly PDE2 and PDE3, can work as cGMP effectors as they provide a mechanism for cGMP-to-cAMP cross-talk (**Figure 1**). cGMP signals triggered by NO and/or natriuretic peptides control the activity of distinct cell types in numerous tissues and organs including the cardiovascular and nervous system. In addition to classical short-term effects like smooth muscle relaxation and platelet inhibition (Feil et al., 2003), cGMP regulates long-term processes from learning and memory (Kleppisch & Feil, 2009) to tissue remodelling during bone growth (Teixeira et al., 2008) and atherogenesis (Lehners et al., 2018).

The critical role of the NO-GC and natriuretic peptide/GC-A systems in the regulation of arterial blood pressure has been known for a long time. Recent studies illustrated that genetic variants in components of this pathway (*e.g.*, NO-GC, ANP, BNP, PDEs) significantly influence blood pressure and cardiovascular disease risk in humans (Ehret et al., 2011; Erdmann et al., 2013; Maass et al., 2015). Genetic variants of NO-GC and cGK type I found in humans have been causally linked to altered vascular structure and remodelling (Guo et al., 2013; Herve et al., 2014). A genetic predisposition to enhanced NO/cGMP signalling is associated with reduced risks of coronary heart disease, peripheral arterial disease, and stroke, but also with an increased risk of hay fever and possibly colorectal cancer (Dang et al., 2020; Emdin et al., 2018). Surprisingly, however, a recent study indicated that cardiovascular disease is associated with higher plasma cGMP levels (Zhao et al., 2020).

Drugs that increase the cGMP concentration are successfully used in the clinic. NO-releasing organic nitrates such as glycerol trinitrate are used for the treatment of angina pectoris, and PDE5 inhibitors such as sildenafil for erectile dysfunction and pulmonary hypertension. More recently, the GC-C agonist linaclotide has been approved for the treatment of chronic idiopathic constipation and irritable bowel syndrome, the NO-GC stimulator riociguat for certain forms of pulmonary hypertension, and a combination drug consisting of valsartan (an angiotensin receptor blocker) and sacubitril for use in patients with heart failure with reduced ejection fraction (HFrEF) (McMurray et al., 2014). Sacubitril inhibits the natriuretic peptide-degrading endopeptidase neprilysin, thereby elevating intracellular cGMP levels. In 2019, treatment with the CNP analogue vosoritide was reported to result in a sustained increase of bone growth in children with achondroplasia (Savarirayan et al., 2019). In 2020, it was shown in the phase III VICTORIA trial that the NO-GC stimulator vericiguat reduced the risk of heart failure hospitalization or cardiovascular death in HFrEF patients, when given in combination with available heart failure therapies (Armstrong et al., 2020b). However, in patients with heart failure with preserved ejection fraction (HFpEF) and recent decompensation, treatment with vericiguat did not improve the physical limitation score compared with placebo (Armstrong et al., 2020a). These findings indicate that the NO/cGMP pathway is operative in the progression of HFrEF, but not HFpEF.

NO-GC stimulators are currently tested in clinical studies for a number of indications, such as hypertension, chronic kidney disease, fibrotic diseases, sickle cell disease, neuroprotection, and dementia (Sandner, 2018). Preclinical studies indicate numerous additional potential applications of cGMP-elevating drugs. For instance, drugs that activate NO-GC may reduce bone loss in postmenopausal women (Joshua et al., 2014), post-infarct heart damage (Frankenreiter et al., 2017), and obesity (Hoffmann et al., 2015). PDE5 inhibitors may protect from noise-induced hearing loss (Jaumann et al., 2012) and improve anti-tumour immunity in various cancers (Meyer et al., 2011; Serafini et al., 2006). On the other hand, drugs that enhance cGMP signalling could have unexpected side effects. For example, prolonged pharmacological stimulation of cGK activity may cause increased oxidative stress and vascular damage (Guo et al., 2013; Schwaerzer et al., 2019). Of note, it has been reported that men taking sildenafil have a significantly increased risk of melanoma (Li et al., 2014) and preclinical studies discovered a growth-promoting cGMP pathway in melanoma cells (Arozarena et al., 2011; Dhayade et al., 2016). These findings call for more well-controlled prospective clinical studies to confirm a causal relationship between PDE5 inhibitors and melanoma risk.

The results described above demonstrate that cGMP is central to human physiology, pathophysiology, and pharmacotherapy. However, it is far from clear how the positive and negative effects of cGMP signalling and cGMP-elevating drugs arise at the molecular, cellular, and organismal level. In the present review article, we will summarise how visualisation of cGMP in real time in living cells has contributed to an improved understanding of this versatile signalling system. We will discuss recent technological and biological advances, with a focus on cGMP imaging in cells of the cardiovascular system, such as vascular smooth muscle cells

(VSMCs), cardiomyocytes, cardiac fibroblasts, and platelets. For lack of published cGMP imaging data, endothelial cells and pericytes are not included. The development of genetically encoded cGMP indicators and their applications have been summarised in several previous reviews. These articles cover cGMP imaging in multiple cell types and tissues (Russwurm & Koesling, 2018) or focus on cardiomyocytes (Cuello & Nikolaev, 2020), neurons (Gorshkov & Zhang, 2014), or technical aspects (Thunemann et al., 2013a).

2. Basics of cGMP imaging

2.1. Why is cGMP imaging useful?

As outlined above, the molecular and cellular mechanisms that underlie cGMP's multiple roles in (patho-)physiology and therapy are not well understood. For instance, it is not always clear which cell types mediate the effects of cGMP observed in preclinical animal models or human genetic and pharmacological studies, such as blood pressure regulation, cardioprotection, or tumour inhibition. At the single-cell level, it is assumed that subcellular changes in the cGMP concentration are important for signalling specificity (Fischmeister et al., 2006). Distinct cGMP compartments or microdomains may be established by the interplay between cGMP generation and degradation via specific guanylyl cyclases and PDEs, respectively, combined with physical barriers that limit cGMP diffusion. Such structural barriers could be present in zones, where the cytoskeleton or the endoplasmic reticulum approaches the plasma membrane. It is a major challenge in cGMP research to improve our knowledge of intracellular cGMP compartments and their functional relevance. The ability to visualise when, where and how much cGMP is produced in an individual cell under normal and pathological conditions or during pharmacotherapy with cGMP-elevating drugs would greatly improve our understanding of cGMP signalling.

cGMP can be detected via antibody-based methods (**Figure 2a**, left). Commercially available enzyme-linked immunosorbent assays (ELISAs) for cGMP are probably most widely used. Because these assays are performed with extracts of homogenised cells and tissues, they have low temporal and no spatial resolution. To provide spatial information, antibodies for immunohistochemical detection of cGMP in fixed tissue sections have been developed (de Vente et al., 1987). In any case, antibody-based methods are not able to monitor dynamic cGMP signals in real time in an individual cell under different conditions. Moreover, cGMP levels determined by ELISA or immunostaining may not always accurately reflect the situation in living cells. For instance, in many studies, cGMP is determined in the presence of the broad-spectrum PDE inhibitor IBMX to prevent potential cGMP degradation and signal loss during sample preparation. However, cGMP concentrations determined after incubation of cells with IBMX should be interpreted with caution with respect to their relevance for cGMP signalling in the presence of physiological PDE activity.

To overcome the limitations of conventional cGMP measurements, genetically encoded fluorescent cGMP indicator proteins are being developed for two decades, with the ultimate goal to allow us 'watching' cGMP signals as they happen in a native healthy environment as

well as under conditions of disease and pharmacotherapy. Importantly, this allows acute cGMP signalling to be observed after application of different ligands and drugs in single cells and, thus, can reveal cell-to-cell variability of cGMP signalling in cell populations (**Figure 2a**, right).

2.2. cGMP biosensors and transgenic cGMP sensor mice

Genetically encoded fluorescent biosensors based on variants of the green fluorescent protein (GFP) are powerful tools for real-time imaging of cGMP in native cells. Their common working principle is that they change their fluorescence upon binding of cGMP. When expressed in a cell or tissue, the sensor's change of fluorescence can be directly monitored under a microscope in real time, for example, after application of a natriuretic peptide or a NO-releasing compound. All currently used cGMP sensors contain one or two cGMP-binding sites derived from a cGK or PDE5 fused to GFP-like fluorophores, and many of them detect cGMP by the principle of Förster/fluorescence resonance energy transfer (FRET). Depending on the sensor, cGMP binding induces either an increase or a decrease of the FRET efficiency. **Figure 2b** and **Movie S1** illustrate FRET-based cGMP imaging with cGi-500, a so-called cGi-type biosensor that shows decreased FRET upon cGMP binding. Details about these biosensors and the conduction of a typical FRET/cGMP imaging experiment are given in the associated figure legend.

cGMP can also be detected with non-FRET-based fluorescent indicators such as FlincG (fluorescent indicator of cGMP). FlincG is composed of a single circularly permutated GFP fused to the regulatory domain of cGK type I containing two cGMP-binding pockets; the sensor responds to cGMP binding with an increase in GFP fluorescence intensity (Bhargava et al., 2013; Nausch et al., 2008). A similar intensiometric cGMP indicator, Green cGull, is based on a cGMP-binding domain of the PDE5 inserted into the GFP-like citrine protein (Matsuda et al., 2017).

Table 1 lists some criteria that a cGMP biosensor should fulfil to be useful for real-time monitoring of the spatial and temporal pattern of cGMP signals in intact cells. A recent overview of available cGMP indicators and their biochemical key features can be found elsewhere (Russwurm & Koesling, 2018). Currently, the FRET-based sensors of the cGi family, particularly cGi-500 (Russwurm et al., 2007), are frequently used for cGMP imaging. These sensors comprise the tandem cGMP-binding sites of cGK type I flanked by CFP and YFP. According to their cGMP affinity, they are named cGi-500, cGi-3000, and cGi-6000 (for 'cGMP indicator' with an apparent EC_{50} of 500 nM, 3000 nM, and 6000 nM, respectively). The cGi-type sensors are useful for cGMP imaging with respect to speed, amplitude, and reversibility of the cGMP-induced FRET change as well as sensitivity to cGMP and cross-activation by cAMP (Russwurm et al., 2007). Although it is difficult to predict the basal and stimulated cGMP concentrations in each cell *in vivo*, the wide range of cGMP affinities of cGi sensors should allow the detection of physiologically relevant cGMP levels across multiple cell types and tissues.

An important advance for cGMP imaging was the availability of transgenic mice expressing cGMP sensors. In 2013, Thunemann et al. reported the generation of cGMP sensor mouse lines expressing the cGi-500 sensor (Thunemann et al., 2013b). They used a targeted knock-in strategy into the Rosa26 locus combined with Cre/lox–activatable expression of the sensor driven by the ubiquitous cytomegalovirus early enhancer/chicken β-actin/β-globin (CAG) promoter. These mouse lines allow expression of cGi-500 either globally in all tissues or, by crossbreeding of the 'floxed' sensor mouse with Cre mice, in a cell type-specific manner. A detailed characterisation of the global cGMP sensor mouse line showed proof-of-principle that it can be used in diverse imaging scenarios (Thunemann et al., 2013b). In primary smooth muscle cells isolated from sensor mice, robust cGMP signals could be recorded in response to NO, natriuretic peptides, and PDE inhibitors. Excitingly, NO-induced cGMP transients could also be monitored in blood vessels of the isolated retina *ex vivo* as well as of the cremaster and skin in anaesthetised sensor mice *in vivo*. Real-time FRET imaging of cGi-500 transgenic cells allowed the detection of cGMP concentrations in the presumably physiological range between approx. 100 nM and 3 µM. The cGMP concentrations measured by FRET-based imaging correlated well with those determined by ELISA. Importantly, cGi-500 can be broadly expressed *in vivo* without obvious side effects, at least under baseline conditions, or toxicity that might have been expected due to possible buffering of cGMP (Thunemann et al., 2013b). Thus, the cGi-500 sensor mouse is useful to (1) obtain sensor expressing primary cells of basically any cell type for *in vitro* culture and imaging, (2) isolate organs and tissues for *ex vivo* imaging, and (3) perform intravital cGMP imaging *in vivo* in anaesthetised mice (**Figure 3**). Recently, a ubiquitously expressing cGi-500 Rosa26 knock-in mouse line that is very similar to the line originally described by Thunemann and colleagues was published by Menges et al. (Menges et al., 2019).

In 2014, a transgenic mouse line was generated that expresses the cGMP indicator red cGES-DE5 under the control of the α-myosin heavy chain (αMHC) promoter in cardiomyocytes (Götz et al., 2014). Red cGES-DE5 is a FRET-based sensor consisting of a single PDE5-derived cGMP-binding domain, which is flanked by T-Sapphire and the red fluorescent dimer2 (Niino et al., 2009). Compared to most other cGMP indicators, it has a relatively low cGMP EC_{50} of ≈40 nM. The αMHC-red cGES-DE5 mice are used for cGMP imaging in isolated cardiomyocytes. The red cGES-DE5 sensor might be superior to cGi-500 or other cGMP sensors with detection limits >100 nM cGMP, if one expects very low cGMP concentrations in the cells of interest, for instance, in adult cardiomyocytes (see section 3.4.). Similar to the results with cGi-500 transgenic mice, cGMP responses measured by FRET were comparable to antibody-based cGMP detection, and constitutive expression of red cGES-DE5 in mouse hearts did not lead to overt phenotypic abnormalities (Götz et al., 2014). More recently, the same group generated transgenic mice expressing a modified version of red cGES-DE5, pmDE5, under control of the αMHC promoter in cardiomyocytes (Subramanian et al., 2018). The pmDE5 sensor is targeted to caveolin-rich membrane domains including cardiomyocyte T-tubules and might be useful to study membrane-associated cGMP signals.

The following sections summarise recent cGMP imaging studies in cells of the cardiovascular system. Many of these studies used FRET-based biosensors and transgenic cGMP sensor mice. They led to important conceptual advances in cGMP signalling that would have been difficult to obtain with conventional end-point methods for cGMP detection.

3. cGMP imaging in cells of the cardiovascular system

3.1. Heterogeneity of cGMP signalling

It is commonly accepted that cGMP is critical for the maintenance of cardiovascular homeostasis via effects on multiple cell types. For instance, cultured VSMCs express NO-GC, GC-A, and GC-B and, thus, their intracellular cGMP concentration can be increased by NO, ANP, and CNP. Platelets express high amounts of NO-GC and show robust NO-induced cGMP signals but are unresponsive to ANP and CNP. The classical physiological effects of an increase of cGMP in VSMCs and platelets are vasodilation and inhibition of thrombosis, respectively. In contrast, the expression and functional significance of components of the cGMP pathway in cardiomyocytes is a matter of debate (Lukowski et al., 2014; M. Zhang & Kass, 2011). It is not clear whether the beneficial cGMP-induced effects on cardiac pathologies like heart failure are mediated via actions of cGMP in cardiomyocytes and/or other cells such as cardiac fibroblasts, VSMCs, endothelial cells, and platelets.

It is known that application of NO and natriuretic peptides to the same cell population or tissue can lead to differential effects. For instance, a study with human epithelial cells reported that stimulation of GC-A with BNP inhibited Ca^{2+} efflux via the plasma membrane, while activation of NO-GC with an NO donor increased reuptake of Ca^{2+} into intracellular stores without affecting Ca^{2+} efflux (Zolle et al., 2000). Another study suggested that stimulation of GC-A with ANP relaxes porcine airway smooth muscle strips by decreasing the intracellular Ca^{2+} concentration, whereas activation of NO-GC with NO lowers both the intracellular Ca^{2+} concentration and the Ca^{2+} sensitivity of contraction (Rho et al., 2002). In adult mouse ventricular myocytes, both CNP and NO inhibited myocyte contraction, but only CNP decreased the amplitude of Ca^{2+} transients, suggesting that cGMP generated by NO-GC may decrease myofilament Ca^{2+} sensitivity (Su et al., 2005). In failing rat ventricular muscle strips and myocytes, NO and CNP differentially affected contractility mediated by different G-protein coupled receptors (Afzal et al., 2011), and activation of the BNP/GC-A and CNP/GC-B pathways had markedly different effects on contractility and Ca^{2+} regulation (Moltzau et al., 2014), suggesting different roles of NO and the two natriuretic peptides in the failing heart.

The finding that NO, ANP/BNP, and CNP can regulate distinct cellular functions via different targets in the same cell population or tissue can be explained by at least three possibilities, which we will discuss in the following sections: (1) cGMP-independent mechanisms; (2) cGMP compartmentation in individual cells; (3) heterogeneity of cGMP signalling between individual cells of the tested cell population or tissue. First, NO donors and/or natriuretic peptides may have additional actions on cellular functions, such as Ca^{2+} regulation and contractility, which are not mediated by cGMP. This issue can be addressed by the analysis of knockout cells and tissues lacking the respective cGMP generators. Second, if one assumes that the differential effects of NO and natriuretic peptides are mediated by elevations of cGMP in the same cell, then inhomogeneities in intracellular cGMP distribution arising from different sources (*e.g.*, NO-GC *vs.* GC-A or GC-A *vs.* GC-B) may affect its mechanism of action. The concept of spatial encoding of cGMP signals, or cGMP compartmentation, requires that cGMP levels are high enough to activate effectors (*e.g.*, cGKs) in one region of the cell, but not in others.

Compartmentation could be achieved by physical containment of cGMP molecules, local formation of protein signalling complexes, and selective depletion of cGMP via, *e.g.*, PDEs (Arora et al., 2013; Rich et al., 2014). The actual mechanisms behind the establishment of putative cGMP microdomains largely remain a mystery and direct proof for their existence is scarce. Nevertheless, cGMP compartmentation is an interesting concept consistent with many experimental findings. It is widely accepted that subcellular cGMP microdomains play important roles in cardiovascular cells and that they are dysfunctional in disease and can be restored by cGMP-elevating drugs (Cuello & Nikolaev, 2020; Preedy et al., 2020) (**Figure 1**). A third possibility is that differential effects of NO, ANP/BNP and CNP that we observe in a population of cultured cells or in a tissue are related to differences in the expression pattern of cGMP pathway components between individual cells of the analysed population. Thus, a major question in cGMP research is whether differential cGMP responses are due to heterogeneity of downstream signalling *within* the same cell (compartmentation) or *between* individual cells (cell-to-cell variability) of the analysed population? Single-cell cGMP imaging is a powerful technique to address this question (see section 4.).

Initial studies on subcellular cGMP pools were performed by recording the activity of overexpressed CNGA2 channels. Because these cation channels are localised at the plasma membrane and activated by cGMP ($K_{1/2}$ ≈2 µM) and cAMP ($K_{1/2}$ ≈40 µM), they can be used as reporters of submembrane cGMP/cAMP signals. In 2006, Piggott et al. used this approach to examine passaged rat VSMCs (Piggott et al., 2006). They found that application of ANP increased CNG channel activity more rapidly than the NO donor SNAP, even when cells were pre-treated with the broad-spectrum PDE inhibitor IBMX. Interestingly, when total cGMP levels were measured by an immunoassay, SNAP triggered stronger cGMP accumulation than ANP. These data suggested that in cultured VSMCs, cGMP produced by GC-A is present close to the plasma membrane, whereas cGMP generated by NO-GC is excluded from these near-membrane regions. The failure of IBMX to increase NO-induced CNG channel activity argues against an important role of PDEs in the apparent segregation of cGMP signals in VSMCs. Also in 2006, Castro et al. used the method based on CNG channels to analyse cGMP compartmentation in isolated cardiomyocytes. Similar to the observations with VSMCs, adult rat cardiomyocytes showed clear activation of CNG channels upon application of ANP or BNP, while several NO donors had little effect. In contrast to VSMCs, PDE activity, specifically PDE5, appeared to prevent cGMP produced by NO-GC to reach the submembrane space. PDE5 was, however, not involved in hydrolysis of GC-A-derived near-membrane cGMP, which was entirely degraded by PDE2 (Castro et al., 2006). Later, the same group used the CNG channel method to show that the different cGMP pools are also subject to regulation via a feedback loop. Activation of cGK limited accumulation of NO-GC-derived cGMP via PDE5 stimulation and increased cGMP produced via ANP/GC-A (Castro et al., 2010). The studies using CNG channel reporters in cultured VSMCs and cardiomyocytes were among the first to suggest that cGMP signals are spatially segregated within cells. In the past 15 years, fluorescence-based biosensors were increasingly used to investigate cGMP dynamics in cardiovascular cells. The major results of these studies are discussed below and summarised in **Table 2.**

3.2. cGMP imaging in VSMCs

The Dostmann group used cGMP indicators to study the spatiotemporal dynamics of NO- and ANP-induced cGMP formation in primary and low passage rat aortic VSMCs. Using cygnet-2.1, one of the first available FRET-based cGMP indicators (Honda et al., 2001), they found that NO donors elicited transient cGMP responses governed by the concerted action of NO-GC and PDE5 (Cawley et al., 2007). These cGMP signals could be triggered repeatedly without apparent desensitization, which is in accordance with the phasic nature of smooth muscle activity. Similarly, imaging with FlincG revealed transient global cGMP elevations to presumably physiological NO concentrations in the low nM range (Nausch et al., 2008). In the same study, ANP triggered sustained submembrane cGMP signals, which spread throughout the cell when PDE5 was inhibited with sildenafil. These data suggested that NO and natriuretic peptides initiate the formation of distinct cGMP pools in VSMCs. In 2012, Held & Dostmann found that cGMP elevations measured with FlincG in primary VSMCs and relaxations of small resistance-type arteries were both sensitive to sub-nanomolar concentrations of NO (Held & Dostmann, 2012). Thus, small changes in the concentration of NO can have strong effects on the cGMP level in VSMCs, which also governs the dynamic regulation of vascular tone.

In 2013, Sinha et al. used cygnet-2.1 to analyse cGMP export via the multidrug resistance-associated protein 4 (MRP4), which transports both cAMP and cGMP out of the cell, and showed that it plays a role in the regulation of fibroblast cell migration (Sinha et al., 2013). Soon thereafter, Krawutschke and colleagues investigated the role of MRP4-mediated cGMP export in mouse primary aortic VSMCs infected with a cGi-6000-encoding adenovirus (Krawutschke et al., 2015). First, they measured intra- and extracellular cGMP levels by immunoassay and found that after application of high concentrations of the NO donor GSNO (1 mM) a substantial amount of cGMP was transported out of the cell via MRP4. These results were complemented by FRET-based imaging, which revealed a potentiation of intracellular cGMP signals induced by low concentrations of GSNO (<100 nM) in the presence of the MRP4 inhibitor MK571. In the same study, the authors performed imaging with cGi-6000 to characterise cGMP breakdown via PDEs and potential mechanisms of cAMP/cGMP cross-talk in VSMCs (Krawutschke et al., 2015). PDE5 was the major PDE responsible for reducing NO-, ANP-, and CNP-induced cGMP signals. In addition, cGMP stimulated by natriuretic peptides, but not NO, was degraded by PDE3, consistent with the membrane association of PDE3 in smooth muscle (Rybalkin et al., 2003). The PDE1 inhibitor vinpocetine did not affect cGMP signals. Interestingly, isoproterenol-induced cAMP elevations apparently also triggered cGMP increases, which were not caused by competition of cAMP with cGMP degradation by PDE3, but perhaps by competition for export. These findings indicated that cGMP export in VSMCs is an important regulator of vascular relaxation. In addition, MRP4-mediated export of cyclic nucleotides might provide a mechanism for cAMP-to-cGMP cross-talk and *vice versa* and, because it should lead to a local decline of the cGMP concentration in submembrane regions, also for cGMP compartmentation.

CNG channel-based recordings of cGMP in passaged human VSMCs also revealed major roles of PDE5 and PDE3 for cGMP hydrolysis (Feiteiro et al., 2016). However, in contrast to the study of Krawutschke et al. who used FRET-based cGMP imaging with cGi-6000 in primary mouse VSMCs, PDE3 appeared to target not only cGMP formed by natriuretic peptides, but also NO-induced cGMP. These findings indicate that certain aspects of cGMP signalling might differ in mouse *vs.* human VSMCs and/or in primary/contractile *vs.* passaged/synthetic VSMCs, perhaps because they express different levels of proteins of the cGMP pathway. Alternatively, the different results regarding the role of PDE3 might be due to technical differences. If we consider that NO increases cGMP primarily in the cytosol and PDE3 is bound to the plasma membrane, we would indeed expect that the amount of NO-induced cGMP reaching the membrane-associated imaging probe (CNG channel) used by Feitero et al. is affected by PDE3 activity, while the amount of NO-induced cGMP detected by the cytosolic probe (cGi-6000) used by Krawutschke et al. should only be slightly altered by PDE3 inhibition.

In 2019, Zhang et al. studied cGMP degradation by PDE1, PDE5, and PDE9 in passaged rat VSMCs expressing cGi-500 (Zhang et al., 2019). Like the previous studies, they found a major role for PDE5 in hydrolysing cGMP produced by NO and natriuretic peptides. However, in contrast to the findings of Krawutschke and colleagues, PDE1 appeared to be clearly involved in the degradation of cGMP, particularly of CNP-induced cGMP. This difference might be attributable to the use of primary *vs.* passaged VSMCs and/or different PDE1 inhibitors. In addition, Zhang and colleagues observed a role of PDE9 in targeting NO-induced but not ANP- or CNP-induced cGMP signals (Zhang et al., 2019), contrasting with neonatal cardiomyocytes, in which PDE9 was reported to regulate ANP- rather than NO-stimulated cGMP signals (Lee et al., 2015). Long-term pharmacological inhibition of PDE1, PDE5 and PDE9 under basal and cGMP-stimulating conditions led to relatively mild and inconsistent effects on the growth of passaged VSMCs, making it difficult to draw strong conclusions about the specific functional roles of these PDEs in vascular cell growth and survival (Zhang et al., 2019).

In the past years, VSMCs isolated from cGi-500 sensor mice have been successfully applied in a number of additional studies, for instance, to show that oxidative stress activates cGK type I in a cGMP-independent manner (Müller et al., 2012) and to validate the functionality of a novel RNA-based cGMP sink (Kröner et al., 2014) as well as a new caged NO donor for light-triggered NO/cGMP signalling (Stroppel et al., 2018).

3.3. cGMP imaging in neonatal cardiomyocytes

Early imaging experiments with cygnet-2.1 provided evidence that neonatal rat ventricular myocytes can generate cGMP in response to NO and that PDE5 inhibition with sildenafil enhances cGMP in these myocytes (Takimoto et al., 2005). Using the same sensor, another study confirmed NO-induced cGMP synthesis in neonatal rat ventriculocytes and showed that norepinephrine increases the cGMP content in these myocytes, presumably via stimulation of β_3-adrenoceptors and activation of NO synthase (Mongillo et al., 2006). Stangherlin et al. used real-time FRET imaging of neonatal rat ventriculocytes expressing targeted cAMP and cGMP

biosensors to detect cyclic nucleotides at specific locations (Stangherlin et al., 2011). To detect cGMP in subcellular compartments where protein kinase A normally resides, they fused the cygnet-2.1 cGMP sensor to the dimerization and docking domains of two different protein kinase A regulatory subunits, RI and RII, resulting in RI_cygnet-2.1 and RII_cygnet-2.1. While activation of NO-GC with SNAP generated a comparable rise of cGMP detected by RI_cygnet-2.1 and RII_cygnet-2.1, stimulation of GC-A with ANP resulted in a significantly larger cGMP signal in the RII than RI compartment. Moreover, by using targeted and nontargeted cAMP sensors, the authors measured the local and global cAMP responses to adrenergic stimulation with isoproterenol. Interestingly, local but not global cAMP signals were profoundly affected by cGMP, presumably via local cGMP-regulated PDE2 and PDE3 activities (Stangherlin et al., 2011). This study provided evidence for a compartmentalised cGMP-to-cAMP cross-talk that might regulate catecholamine-dependent processes in cardiomyocytes.

Recordings with FlincG revealed that both NO and ANP augmented cGMP levels in neonatal rat cardiomyocytes and that NO- and ANP-induced cGMP were under control of PDE5 and PDE9, respectively (Lee et al., 2015). Recently, cGMP imaging in neonatal mouse ventricular myocytes was reported using the cytosolic cGi-500 sensor as well as new modified versions of it targeted to the plasma membrane (PM-cGi-500) and outer mitochondrial membrane (OMM-cGi-500) (Brescia et al., 2020). Stimulation of NO-GC resulted in responses of all three sensor constructs, suggesting that cGMP formed by NO-GC is globally distributed in neonatal cardiomyocytes including to the near-membrane regions. Moreover, the authors found that in neonatal myocytes PDE2 is more important for cGMP degradation than PDE5.

3.4. cGMP imaging in adult cardiomyocytes

While cGMP sensors like cygnet-2.1, FlincG, and cGi-500 (cGMP EC_{50} ≈2 µM, ≈0.2 µM, and ≈0.5 µM, respectively) were successfully used to measure cGMP in neonatal rat cardiomyocytes, cGMP imaging in adult cardiomyocytes turned out to be challenging, presumably because these cells have very low cGMP concentrations (Cuello & Nikolaev, 2020; Russwurm & Koesling, 2018). To overcome this problem, the Nikolaev group has introduced the use of a more sensitive cGMP indicator, red cGES-DE5 (cGMP EC_{50} ≈0.04 µM), initially developed by Niino and colleagues (Niino et al., 2009). Nevertheless, in adult cardiomyocytes isolated from red cGES-DE5 transgenic mice, cGMP elevations upon application of NO donors or ANP were very weak to undetectable, while CNP induced robust cGMP signals (Götz et al., 2014). Similar results were obtained in a study with adult myocytes from transgenic mice expressing the cGi-500 sensor (Menges et al., 2019). In this study, neither NO nor several NO-GC stimulators or activators induced a detectable cGMP increase, even in the presence of IBMX. ANP was also ineffective, but CNP induced strong cGMP elevations. Experiments with PDE inhibitors revealed that the CNP-induced cGMP was degraded via PDE2 and PDE3 but not PDE1, PDE5, or PDE9 (Menges et al., 2019). The failure to detect cGMP changes upon application of NO and ANP as well as PDE5 and PDE9 inhibitors in adult cardiomyocytes contrasts with previous findings obtained in neonatal cardiomyocytes (see section 3.3.). Thus, it appears that cGMP signalling differs fundamentally

between neonatal and adult cardiomyocytes, with neonatal myocytes expressing ANP/GC-A and NO/NO-GC pathways, whereas adult myocytes express predominantly the CNP/GC-B pathway.

The apparent absence of GC-A and NO-GC activities in adult cardiomyocytes contrasts with studies that have described phenotypes of Cre/lox-mediated cardiomyocyte-specific knockouts of GC-A (Holtwick et al., 2003) and NO-GC (Frankenreiter et al., 2018), or detected NO-GC in adult rodent cardiomyocytes by immunostaining (Schobesberger et al., 2020; Tsai et al., 2012). These contradictory results could be related to technical issues, for instance, the use of Cre mice that produce unexpected gene knockouts also in non-cardiomyocytes (Skryabin et al., 2004) or the use of antibodies with unknown specificity. Alternatively, although cGMP imaging has proven to be a very sensitive method for the detection of cGMP signals, it cannot be excluded that functionally relevant cGMP formed in a cell is either locally restricted to regions not accessible to the biosensor or remains below the detection limit. Indeed, in adult rodent cardiomyocytes, cGMP signals elicited by ANP appeared to be detectable with membrane-bound CNG channels (Castro et al., 2010; Castro et al., 2006) but not cytosolic cGMP indicators (Götz et al., 2014; Menges et al., 2019).

A strategy to enhance the detection of presumably tiny submembrane cGMP signals is the local stimulation of guanylyl cyclases combined with highly sensitive cGMP indicators. Subramanian et al. isolated ventricular cardiomyocytes from adult red cGES-DE5 mice and stimulated them with CNP and ANP locally at defined membrane structures with a precisely positioned scanning nanopipette (Subramanian et al., 2018). Upon application of CNP, far-reaching cGMP signals originating from both T-tubules and cell crests could be detected, while ANP elicited cGMP elevations only when it was applied to T-tubules but not to cell crests. To detect FRET changes, relatively high local ANP concentrations were required (≈700 nM). The ANP-induced cGMP signals were considerably smaller than CNP-triggered signals and, interestingly, strictly confined to the site of stimulation. However, inhibition of PDE2 transformed the local ANP signals to far-reaching cGMP signals (Subramanian et al., 2018). These results indicated that GC-B is uniformly distributed across the cardiomyocyte plasma membrane and generates global cGMP signals upon stimulation with CNP. In contrast, ANP-induced cGMP signals are apparently spatially restricted to T-tubules by local pools of GC-A and PDE2. This is one of the few studies in which a cGMP microdomain was directly visualised by imaging, albeit only after local stimulation. The functional relevance of these putative cGMP compartments must be shown by further studies.

Very recently, a similar combination of local stimulation and cGMP imaging with red cGES-DE5 was employed to analyse the coupling of β_3-adrenoceptors to NO-GC and production of cGMP in adult rat cardiomyocytes (Schobesberger et al., 2020). In healthy myocytes, β_3-adrenoceptors and NO-GC were found in T-tubules, and isoproterenol induced a β_3-adrenoceptor-dependent increase in cGMP. Heart failure caused a redistribution of the receptors leading to impaired NO/cGMP/PDE2 signalling. The reorganisation of the β_3-adrenoceptor/cGMP signalosome in failing cardiomyocytes was associated with decreased cGMP levels and reduced negative cGMP-to-cAMP cross-talk via PDE2 leading to an increase

in cAMP levels, which might promote pathological cardiac hypertrophy and remodelling. A limitation of this study was that the presence of functional NO-GC was not confirmed by cGMP imaging upon stimulation with an NO donor (Schobesberger et al., 2020).

3.5. Intercellular cGMP transfer in the heart

In 2015, the Jaffe group reported cGMP imaging in live ovarian follicles of cGi-500 sensor mice (Shuhaibar et al., 2015). Interestingly, they observed that after application of luteinising hormone, cGMP began to diffuse via gap junctions from the oocyte into the surrounding granulosa cells, thus, lowering the cGMP concentration in the oocyte and restarting meiosis. This was the first direct visualisation of intercellular cGMP trafficking and it was speculated that cGMP diffusion through gap junctions could provide a general mechanism for diverse physiological processes (Shuhaibar et al., 2015) (**Figure 1**). Indeed, a little later Menges et al. found strong evidence that a similar mechanism is active in cardiac tissue (Menges et al., 2019). Motivated by their inability to detect functional NO-GC in isolated adult cardiomyocytes (see section 3.4.), they came up with the hypothesis that cardiac fibroblasts could be a source of NO-stimulated cGMP in the adult heart. In contrast to myocytes, fibroblasts prepared from the hearts of cGi-500 sensor mice displayed profound NO-induced cGMP elevations. The authors calculated that even small amounts of fibroblasts present in the cardiomyocyte cultures, as regularly present (Kohl, 2003), would suffice to explain the cGMP increases upon stimulation of NO-GC measured by immunoassays in homogenates of myocyte cultures. The cardiac fibroblasts showed also strong cGMP signals to CNP, but marginal responses to ANP. PDE1 was the major PDE responsible for degradation of both NO- and CNP-induced cGMP, with an additional contribution of PDE2 to the hydrolysis of cGMP produced by NO-GC (Menges et al., 2019).

Interestingly, when Menges and colleagues prepared co-cultures of cGi-500-expressing cardiomyocytes with wildtype fibroblasts not expressing cGi-500, they were able to record NO-induced cGMP signals in some of the cardiomyocytes, at least when IBMX was added, and these cGMP elevations were suppressed in the presence of several gap junction inhibitors (Menges et al., 2019). This study demonstrated using *in vitro* models that NO-induced cGMP is formed in cardiac fibroblasts but not myocytes from adult mice, and that cGMP produced in fibroblasts can enter cardiomyocytes via gap junctions. If this fibroblast-to-myocyte coupling can be confirmed in intact heart tissue, then it might also explain at least some of the beneficial effects of NO-GC stimulators on cardiac diseases observed in preclinical *in vivo* models and patients (see section 1.).

3.6. Mechanosensitive cGMP signalling in platelets

NO produced by the vascular endothelium can diffuse not only into VSMCs but also platelets. Although it is generally accepted that activation of NO-GC in platelets results in platelet inhibition, there is increasing evidence that NO-GC-derived cGMP might also be involved in platelet activation (Wen et al., 2017). The multiple functions of cGMP in platelets are not well understood, in part because it was not possible to monitor dynamic cGMP changes in living platelets under conditions that represent the *in vivo* situation regarding platelet interactions with immobilised substrates and shear forces. Recently, the first cGMP imaging experiments in platelets were reported by Wen and colleagues (Wen et al., 2018). They used platelets from cGi-500 sensor mice and subjected them to FRET imaging in a collagen-coated flow chamber

ex vivo. As expected from previous studies (Rondina & Weyrich, 2012), application of NO donors under flow conditions, but not ANP or CNP, led to robust cGMP elevations, which were further potentiated by inhibition of PDE2, PDE3, or PDE5. Surprisingly, when they switched the superfusion off and on in the presence of NO, the authors observed a dramatic decrease and increase of the cGMP signal, respectively. These results indicated that cGMP formation by platelet NO-GC in the presence of NO is strongly enhanced by fluid shear stress (Wen et al., 2018).

Importantly, intravital FRET microscopy in platelet-specific cGMP sensor mice confirmed spatiotemporally dynamic cGMP signals during thrombus formation *in vivo*, with higher cGMP levels in the shear-exposed thrombus periphery than in its core region (**Figure 3c**). Imaging of thrombus formation in platelet-specific NO-GC knockout mice demonstrated the functional relevance of platelet cGMP for thrombus resolution (Wen et al., 2018). This study not only identified a previously unknown shear-dependent NO/cGMP pathway in platelets, but also proposed the novel concept of mechanosensitive cGMP signalling (**Figure 1**). Indeed, preliminary evidence suggests that NO-GC, cGK type I and other yet unidentified proteins form a signalling complex at the plasma membrane, which in the presence of NO converts mechanical force into cGMP signals in platelets, VSMCs, and perhaps many other cell types (Wen et al., 2018; ML and RF, unpublished data 2021).

4. In-cell compartmentation *vs.* cell-to-cell variability of cGMP signalling

To explain receptor-specific responses observed in experiments and how these responses might differ in different regions of a cell, many researchers have proposed cGMP microdomains (**Figure 1**). However, direct visualisation of subcellular cGMP pools appears to be challenging and, as described in the previous sections, to date most of the evidence supporting their existence is indirect. A widely used experimental approach is to measure cGMP levels in individual cells of a given cell population (*e.g.*, isolated VSMCs or cardiomyocytes) upon stimulation of NO-GC, GC-A, or GC-B in the absence and presence of PDE inhibitors. If cGMP signals triggered by NO, ANP, or CNP are differentially affected by the same PDE inhibitor, then this is frequently interpreted as evidence for spatially distinct pools of cGMP produced by NO-GC *vs.* GC-A *vs.* GC-B.

Figure 4a depicts the current model of in-cell compartmentation *vs.* cell-to-cell variability of cGMP signalling and shows a hypothetical experiment, in which cells are treated with ANP and CNP in the absence and presence of a PDE inhibitor, to illustrate the interpretation problem that we might have with such data. To address if differential effects of cGMP-elevating compounds might be related to in-cell compartmentation or cell-to-cell heterogeneity of cGMP generators and effectors, one would like to look at the responses of the *same* individual cell, when it is stimulated consecutively with all agents of interest, *e.g.* with ANP, ANP + PDE inhibitor, CNP, and CNP + PDE inhibitor (**Figure 4a**, cGMP traces). Furthermore, it would be informative to know how many cells of a population have been measured in an experiment and how many of them responded to ANP, CNP or both with a cGMP increase. To date, such

single-cell analyses of cGMP responses are rarely reported, and the issue of cell-to-cell variability has apparently not been widely considered in the cGMP research field. In practice, single-cell measurements would be usually performed with ANP in the absence and presence of PDE inhibitor with one aliquot of cells, and another aliquot of cells would be treated with CNP in the absence and presence of PDE inhibitor. Therefore, the assumption that differential effects of cGMP-elevating agents such as natriuretic peptides and PDE inhibitors occur in the *same* cell should be made with caution until this has been confirmed by appropriate single-cell measurements as shown in **Figure 4a**. Indeed, by recording cGMP in individual cells upon sequential stimulation with ANP and CNP, we observed a marked cell-to-cell heterogeneity of cGMP signalling in cultures of primary VSMCs, with subpopulations of cells responding strongly to ANP but not CNP and *vice versa* (ML and RF, unpublished data 2021).

Surprisingly, although cGMP imaging technology is available for decades, to date few studies have been published that attempted to directly visualise spatially restricted cGMP pools. In one publication, primary VSMCs expressing FlincG were analysed using confocal microscopy (Nausch et al., 2008). The authors reported that application of the NO donor DEA-NO elicited global cGMP elevations, while ANP triggered submembrane increases of cGMP, which were converted to global cGMP elevations upon inhibition of PDE5. In another report, cGMP was monitored by epifluorescence microscopy in adult cardiomyocytes expressing red cGES-DE5 (Subramanian et al., 2018). Here, local application of CNP and ANP evoked global and spatially restricted cGMP signals, respectively, and PDE2 inhibition transformed the local ANP-induced cGMP signals to far-reaching signals. These two studies provided preliminary evidence based on visualisation of cGMP that NO, ANP, and CNP can induce distinct cGMP patterns in cardiovascular cells. In the future, further imaging experiments are needed to confirm these promising findings in isolated cells and intact tissues, in particular to show that different cGMP pools coexist in the same cell.

Based on the relatively low activities of guanylyl cyclases and PDEs combined with fast cGMP diffusion rates, it is unlikely that cGMP microdomains can form without mechanisms that restrict the spatial spread of the molecule (Rich et al., 2014). Recently, two studies identified such mechanisms for the establishment of cAMP nanodomains. Bock et al. observed ‘buffered diffusion’ of cAMP via cAMP-binding sites (Bock et al., 2020), while Zhang and co-workers found that protein kinase A regulatory subunits assemble liquid droplets to further localise cAMP signalling (Zhang et al., 2020). The new model proposes that under basal conditions, most cAMP is bound, and its free concentration is low, especially in nanometer-sized domains created by individual PDE molecules. In these low cAMP nanodomains, cAMP targets are protected from the cyclic nucleotide until receptor stimulation leads to a cAMP increase that floods the nanodomain and leads to downstream effects in this subcellular region (Bock et al., 2020). It is tempting to speculate that similar mechanisms may also form local domains with a decreased cGMP concentration (**Figure 4b**). Note that the new concept predicts that compartmentation establishes *low*, not high, cGMP microdomains.

5. Current challenges and future directions of cGMP imaging

As described in the previous sections, the availability of genetically encoded cGMP indicators has greatly advanced our understanding of cGMP signalling at the level of single living cells. However, there is still room for improvement in the area of cGMP imaging. First, there are some technical issues that can lead to artifacts if not taken care of. The fluorescence of a biosensor can be strongly affected by environmental changes (*e.g.*, pH, solvents, oxidative stress, temperature) or movement of the cell/tissue during the imaging experiment (Rich et al., 2014). Another potential problem is unequal (photo)bleaching of the two fluorophores used for ratiometric imaging. This leads to CFP/YFP ratio changes that do not reflect changes of the intracellular cGMP concentration. True cGMP changes are usually indicated by a divergence of the CFP and YFP emission traces into opposite directions (see **Figure 2b**, middle). In order to avoid the above mentioned pitfalls, FRET/cGMP changes should be validated by appropriate control experiments, for instance, by applying vehicle alone in the absence of cGMP-modulating drug, and one should not only look at the calculated CFP/YFP ratio trace (black in **Figure 2b**, middle) but also at the original CFP and YFP emission traces themselves. To obtain definite proof for the origin of FRET/cGMP signals, cells or tissues from knockout mice lacking the cGMP generator of interest can be imaged under the same conditions as wildtype samples (*e.g.*, Peters et al., 2018; Wen et al., 2018).

An area of major interest in the future is multiplex imaging. It would be exciting to observe separate cGMP compartments in the same cell, or cGMP simultaneously with other signalling molecules such as cAMP or Ca^{2+}. Moreover, with the recent development of genetic biosensors for NO (Eroglu et al., 2018), co-imaging of NO and cGMP appears feasible. To achieve these goals, one could use strategies based on the expression of two fluorescent probes that are spectrally compatible. Indeed, cGMP indicators have been developed that consist of fluorescent proteins other than CFP and YFP. These sensors, *e.g.*, the 'red' sensor, red cGES-DE5 (Niino et al., 2009), or the 'blue' sensor, cygnus (Niino et al., 2010). can be combined with conventional CFP/YFP-based biosensors to measure cGMP and cAMP/Ca^{2+} simultaneously. To directly visualise putative submembrane and global cGMP signals triggered by ANP and NO, respectively, one could co-express a red membrane-targeted and a green cytosolic cGMP sensor and then stimulate the cell consecutively with ANP and NO. For the analysis of cGMP/cAMP cross-talk one could use a red-shifted cGMP sensor together with a green cAMP sensor. Note, however, that at least some of the available green cAMP sensors, such as epac2-camps, appear to respond also directly to NO-induced cGMP elevations precluding their use for the analysis of cGMP/cAMP cross-talk (Krawutschke et al., 2015). Indeed, epac1-camps has been used to measure *cGMP* signals in *Drosophila* motoneurons (Shakiryanova & Levitan, 2008).

Ratiometric and intensiometric cGMP sensors with higher sensitivity, amplitude and dynamic range are under continuous development, for example, FlincG3 (Bhargava et al., 2013), PKG #7 (Ohta et al., 2016), Green cGull (Matsuda et al., 2017), cyGNAL (Betolngar et al., 2019), yellow and red PfPKG (Calamera et al., 2019), or CUTie2 (Klein et al., 2021). Each of these indicators has its strengths and weaknesses and could prove useful for certain

applications. In order to select the best biosensor in each case, it would help to compare them side by side in the same setup with the current 'gold standard', which we believe is cGi-500 (Russwurm et al., 2007). To address questions about subcellular cGMP signalling, sensors should be targeted to the relevant sites, such as the plasma membrane or specific organelles. However, note that fusing a sensor to specific targeting sequences may alter its dynamic range of cGMP detection (Brescia et al., 2020). Because cGMP may also act on membrane targets from outside the cell (Grundy et al., 2018), it would be interesting to generate indicators for extracellular cGMP. In another application, cGMP sensors can be used as reporters for conformational changes of their cGMP-binding domains, *e.g.*, to characterise structural alterations upon binding of cGMP analogues (Valtcheva et al., 2009) or oxidation (Donzelli et al., 2017). In recent years, several other novel tools have been developed that complement cGMP imaging, such as light-regulated cGMP generators and degraders (Tsunoda et al., 2021) and cGMP sinks (Kröner et al., 2014; Ros et al., 2019).

To date, most cGMP imaging studies were performed with isolated cell preparations *in vitro*, such as cultured cardiomyocytes and VSMCs (**Figure 3a**). These studies have undoubtedly provided many novel insights into cGMP signalling, but it is also clear that the expression profile and behaviour of cultured cells can be different from cells in their native three-dimensional tissue environment. For instance, the mobility of membrane receptors can differ strongly between cells embedded in sections of cardiac tissue and isolated cardiomyocytes, presumably because adherent cell cultures have altered membrane properties (Mashanov et al., 2021). Our group observed that cultured VSMCs but not native cells in the healthy vessel wall show strong CNP-induced cGMP elevations (ML and RF, unpublished data 2021). A recent analysis of the adult human heart by single-cell and single-nucleus RNA sequencing highlighted a pronounced cell-to-cell heterogeneity of cardiomyocytes (Litvinukova et al., 2020). Interestingly, the NO-GC β_1 subunit, which is required to form functional NO-GC, seems to be expressed only in a very small population comprising ≈1% of all cardiomyocytes. In addition, GC-B and PDE2, which were clearly detected in adult rodent cardiomyocytes by cGMP imaging (see section 3.4. and **Table 2**), were not detected by RNA sequencing of human adult myocytes. Therefore, we should be careful with translating the findings in cell models to the *in vivo* situation in humans. To complete our picture of cGMP signalling, it appears mandatory to complement the *in vitro* data with results obtained by cGMP imaging in more physiologically relevant samples. Indeed, recent studies demonstrated the feasibility of FRET-based cGMP recordings in whole-mount preparations of the cochlea (Möhrle et al., 2017) (**Figure 3b**) and tibial bones (Shuhaibar et al., 2017) as well as in brain slices *ex vivo* (Betolngar et al., 2019; Giesen et al., 2020; Peters et al., 2018). Even more exciting, using intravital FRET microscopy in anaesthetised cGi-500 sensor mice, we were able to visualise cGMP in small blood vessels during vasodilation (Thunemann et al., 2014; Thunemann et al., 2013b) and in platelets during thrombus formation *in vivo* (**Figure 3c**) (Wen et al., 2018).

To determine which cell types in a tissue respond to endogenous or pharmacological cGMP stimulation, imaging experiments can be performed with respective cell-type specific sensor mice. A Cre/lox-activatable cGi-500 sensor mouse line is available (Thunemann et al., 2013b)

and was already successfully used to generate neuron- and platelet-specific cGMP sensor mice (Peters et al., 2018; Wen et al., 2018). The spectral properties of current CFP/YFP-based sensors limit our ability to probe cGMP in deeper layers of tissues, but the development of far-red biosensors and other refinements of multiphoton FRET microscopy should help to overcome this problem (Shcherbakova et al., 2018; Tao et al., 2015). Imaging of tissues and mice is demanding as it requires animal procedures and advanced equipment such as laser scanning, spinning disk or multiphoton microscopes. The payoff is, however, that these methods enable the correlative analysis of biochemistry, in our case cGMP signals, and (patho-)physiology in a living mammalian tissue or organism.

6. Conclusions

Single-cell cGMP imaging is a powerful tool to study the spatiotemporal dynamics of cGMP signals and their heterogeneity within and between cells of isolated cell populations and intact tissues. This method is also excellently suited to profile the activity of cGMP generators and degraders, or of drugs targeting these proteins, in living cells. In such, cGMP imaging complements traditional antibody-based methods for the detection of components of the cGMP pathway with high specificity and sensitivity. This is of particular value because good antibodies for staining cells or tissue sections are not available for many proteins of the cGMP pathway. The application of biosensors has already greatly impacted our understanding of this signalling system. Studies reviewed in this article have led to exciting, and often unexpected, conceptual advances that would have been difficult to achieve without the use of live-cell cGMP imaging, such as the discoveries of subcellular, intercellular and mechanosensitive cGMP signals (**Figure 1**).

What also emerges from the cGMP imaging studies is that the functional activity of cGMP generators and degraders varies greatly between different cell types of the cardiovascular system. This is summarised in **Table 2**. For instance, populations of both primary and subcultured VSMCs are equipped with all three cGMP generators, NO-GC, GC-A, and GC-B; neonatal cardiomyocytes appear to express NO-GC and GC-A; and adult cardiomyocytes may use predominantly GC-B to produce cGMP. While VSMCs employ mainly PDE5 for cGMP degradation, the major PDE isoforms in rodent cardiac myocytes are PDE2 and PDE3. In addition, each of these cell populations appears to have PDEs that hydrolyse specific cGMP 'pools' induced by NO or natriuretic peptides (**Table 2**). Whether this reflects in-cell compartmentation or cell-to-cell variability of protein expression remains to be demonstrated, for instance, by direct visualisation of cGMP microdomains or by recording cGMP responses in the same cell upon stimulation with different agonists in the absence and presence of a PDE inhibitor.

We anticipate that the next decade of cGMP research will see further refinements of biosensors for multiparameter and deep-tissue imaging and a transition from the analysis of cultured cells *in vitro* to intact tissues *ex vivo* to living animals *in vivo* (**Figure 3**). The correlative visualisation of a biochemical signal, cGMP, with its effects on cell behaviour will certainly

improve our understanding of physiology, pathophysiology, and drug action. Transgenic cGMP sensor mice provide an invaluable resource for sensor-expressing cells and tissues and can be directly used for intravital cGMP imaging, allowing us to watch cGMP signals as they happen under close-to-native conditions. *In vivo veritas* – let's bring light into the darkness with cGMP biosensors!

7. Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY, and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

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Table 1. Properties of useful cGMP indicators

- The structural change upon cGMP binding should be fast and reversible.
- The cGMP-induced change of fluorescence should be strong.
- The sensitivity and selectivity for cGMP should allow the detection of physiological concentrations of cGMP without cross-activation by cAMP; in this regard, cGMP sensors with EC_{50} values for cGMP of ≈1 µM and for cAMP of >50 µM should perform well in most applications.
- Expression of the sensor should not have unwanted side effects such as general toxicity or buffering of intracellular cGMP. The latter may interfere with endogenous cGMP signalling and cause subtle to obvious phenotypes such as altered cell growth and blood pressure. These phenotypes may occur under baseline and/or disease conditions (*e.g.*, heart failure, obesity). To detect cGMP buffering, wild-type and sensor-expressing cells/animals should be compared.
- It should be assured that the localisation of the sensor overlaps with the cellular compartment to be studied, for instance, by using targeted sensor variants.
- The sensor's fluorescence should be inert to environmental changes (*e.g.*, pH, solvents, oxidative stress, temperature) or posttranslational modifications that might occur in a cell.

Table 2. Components of cGMP signalling pathways in cardiovascular cells as determined by cGMP imaging[1,2]

Cell type	cGMP generators[3]	cGMP degraders[3,4,5]	References
VSMCs (cultured)			
Primary VSMCs	NO-GC, GC-A, GC-B	PDE5, PDE3 (GC-A, GC-B) (not detectable: PDE1)	Krawutschke et al., 2015
Passaged VSMCs	NO-GC, GC-A, GC-B	PDE5, PDE1 (GC-B), PDE9 (NO-GC)	Zhang et al., 2019
Cardiomyocytes (cultured)			
Neonatal cardiomyocytes	NO-GC, GC-A	PDE2, PDE3, PDE5 (NO-GC), PDE9 (GC-A)	Takimoto et al., 2005 Stangherlin et al., 2011 Lee et al., 2015 Brescia et al., 2020
Adult cardiomyocytes	GC-B (not detectable: NO-GC, GC-A[6])	PDE2, PDE3 (not detectable: PDE1, PDE5, PDE9)	Götz et al., 2015 Menges et al., 2019 Calamera et al., 2019
Cardiac fibroblasts (cultured)	NO-GC, GC-B (not detectable: GC-A)	PDE1, PDE2 (NO-GC)	Menges et al., 2019
Platelets	NO-GC (not detectable: GC-A, GC-B)	PDE5, PDE2, PDE3	Wen et al., 2018

[1] Note that studies using other detection methods, such as RNA-, antibody- or CNG channel-based detection of pathway components, are not included.

[2] The listed guanylyl cyclases were deduced from the cells' cGMP responses to the respective agonists like NO, ANP, and CNP. The listed PDEs are deduced from the effects of the respective PDE inhibitors on cGMP levels.

[3] If components are not listed, this reflects that they were not tested in the cited studies.

[4] If more PDEs are listed and one of them was found to hydrolyse the major fraction of cGMP, then this PDE is underlined.

[5] If a PDE was reported to degrade preferentially a specific cGMP 'pool', then this is indicated in brackets.

[6] GC-A may generate tiny, localised signals that may require special stimulation and detection methods (Subramanian et al., 2018).

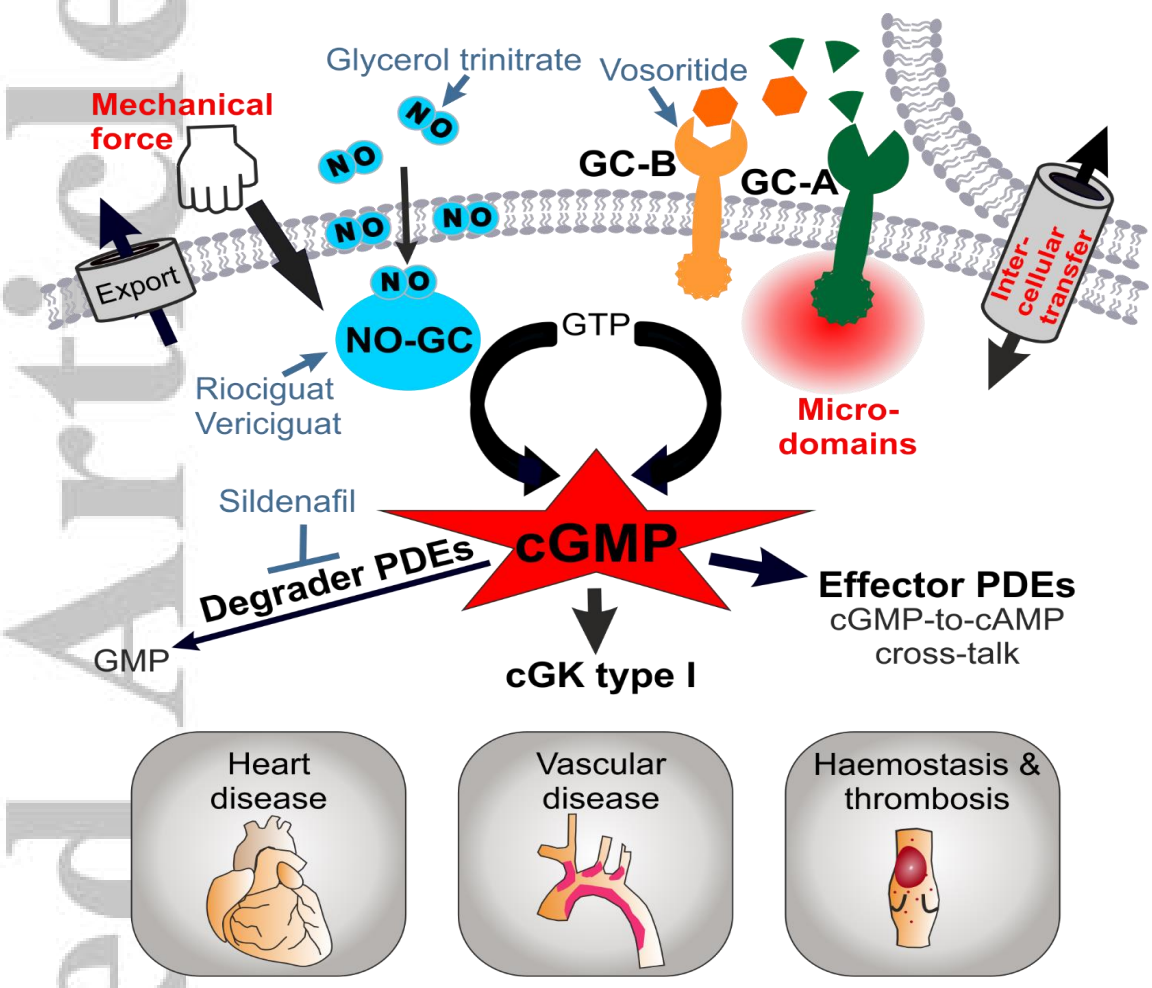


Figure 1. Classical and novel aspects of cGMP signalling in the cardiovascular system. Cardiovascular cells express two types of guanylyl cyclases that generate cGMP from GTP. Intracellular NO-sensitive guanylyl cyclase (NO-GC) is stimulated by binding of the gaseous signalling molecule NO. Transmembrane guanylyl cyclases, such as GC-A and GC-B, are receptors for peptide hormones. GC-A binds atrial and B-type natriuretic peptide (ANP, BNP, green triangles), and GC-B binds C-type natriuretic peptide (CNP, orange hexagons). Once generated by guanylyl cyclases, intracellular cGMP can be removed by active export via multidrug resistance-associated proteins (grey tube, left) or by hydrolysis to GMP via degrader phosphodiesterases (PDEs). Cardiovascular cells express several members of the 11 PDE subfamilies (not shown), which are grouped into cGMP-specific (PDE5, PDE6, PDE9), cAMP-specific (PDE4, PDE7, PDE8) and dual-specificity enzymes degrading both cGMP and cAMP (PDE1, PDE2, PDE3, PDE10, PDE11). The effects of cGMP are mediated by its binding to cGMP-dependent protein kinase type I (cGK type I) and effector PDEs like PDE2 and PDE3, which provide a mechanism for cGMP-to-cAMP cross-talk (not shown). Binding of cGMP to an allosteric site of PDE2 activates cAMP hydrolysis by its catalytic domain, resulting in negative cGMP-to-cAMP cross-talk, *i.e.* an increase of cGMP results in a decrease of cAMP. Conversely, high concentrations of cGMP compete with cAMP at the catalytic site of PDE3, leading to reduced cAMP hydrolysis and, therefore, a positive cGMP-to-cAMP cross-talk. Malfunctions of the cGMP signalling system are associated with cardiovascular diseases (lower boxes). Some drugs (grey blue) that target members of the pathway and are successfully used in the clinics are also indicated. Live-cell cGMP imaging studies discussed in the present review have led to novel concepts (red) such as mechanosensitive cGMP signalling, cGMP microdomains, and intercellular cGMP transfer. For more details, see main text.

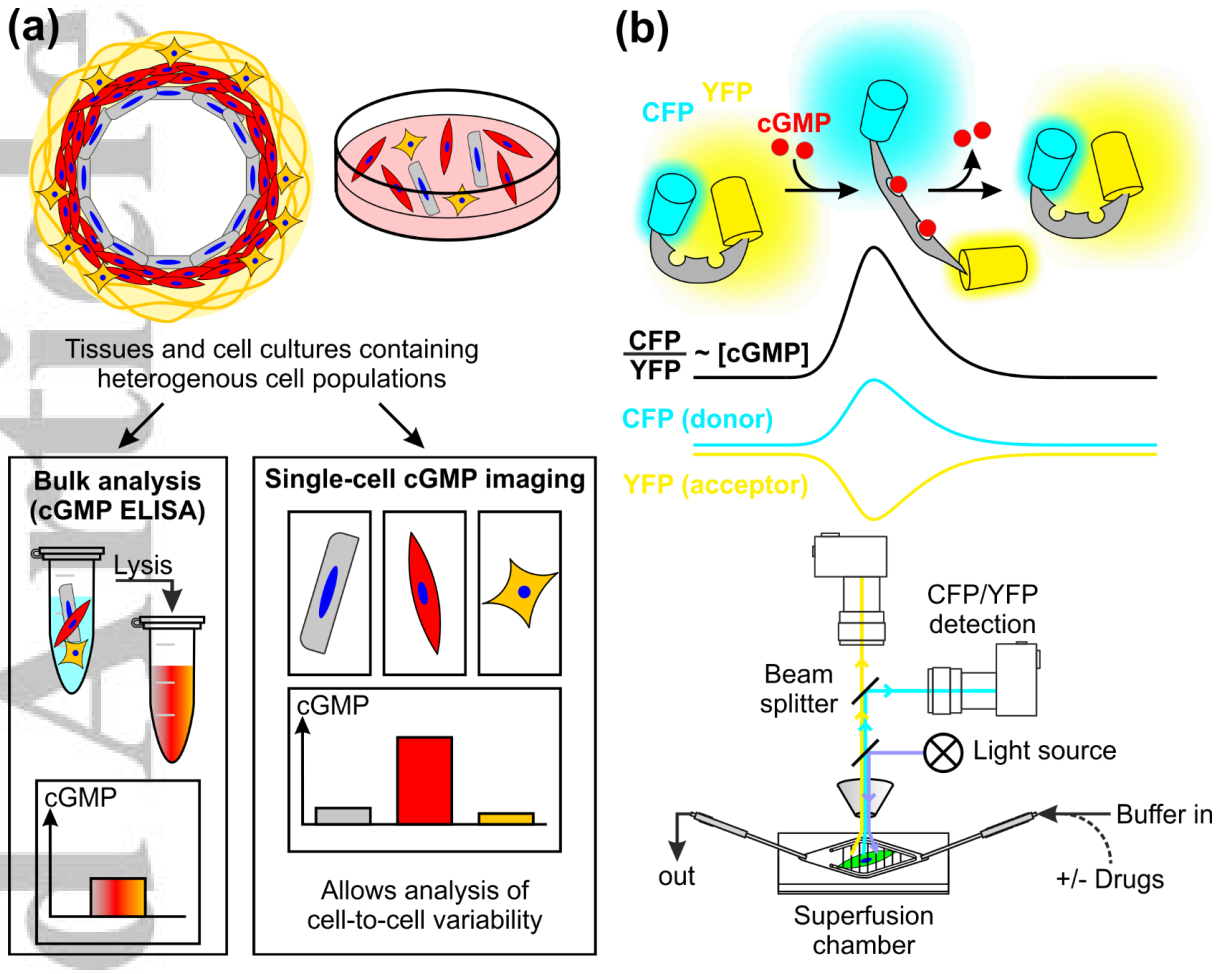


Figure 2. How can we measure cGMP? (a) Comparison of bulk cGMP analysis *vs.* single-cell cGMP imaging. Cell populations in tissues (*e.g.*, blood vessels) and cell cultures are usually heterogenous. Conventional antibody-based cGMP assays such as ELISA (left panel) show the average cGMP concentration of all cells in the sample; information about inter-cellular differences is lost. In contrast, single-cell cGMP imaging (right panel) reveals the cGMP concentration in individual cells and, thereby, allows analysis of cell-to-cell variability. Bars indicate cGMP concentrations in the lysate or in single cells, respectively. Colours match the respective cell populations. Note that even the cells of an apparently 'homogeneous' cell population, *e.g.*, cultured VSMCs or cardiomyocytes, may differ with respect to their expression profile of cGMP signalling proteins. Note also that the cGMP biosensors most likely detect only free cGMP, whereas antibody-based methods detect total cellular cGMP, *i.e.* free cGMP as well as cGMP bound to proteins and other cellular components. **(b)** Operation mode of FRET-based cGMP indicators exemplified with cGi-500 (see also **Movie S1**). cGi-500 consists of CFP (FRET donor, cyan) and YFP (FRET acceptor, yellow) linked by the tandem cGMP-binding domain of cGK type I (grey). FRET relies on the proximity-dependent (≈2-6 nm) energy transfer from the excited donor to the acceptor. Binding of cGMP (red circles) results in a conformational change leading to reduced energy transfer (top). These FRET changes can be depicted via the donor/acceptor emission ratio (CFP/YFP, black trace), which reflects the cGMP concentration change over time (middle). Note that a change in FRET efficiency results in a simultaneous change of the donor and acceptor emission intensities in opposite directions, which can be used for validation purposes. In practice, cells or tissues of interest are placed under a fluorescence microscope (bottom). The FRET donor, CFP, is excited and emissions of both donor and acceptor are recorded simultaneously with the help of a beam splitter. While cells are superfused with buffer, drugs modulating the cGMP signalling pathway can be applied in a timed fashion. As binding of cGMP to the sensor is reversible, various drugs can be tested sequentially on the same sample, allowing the analysis of intra- and inter-cell variability of cGMP responses.

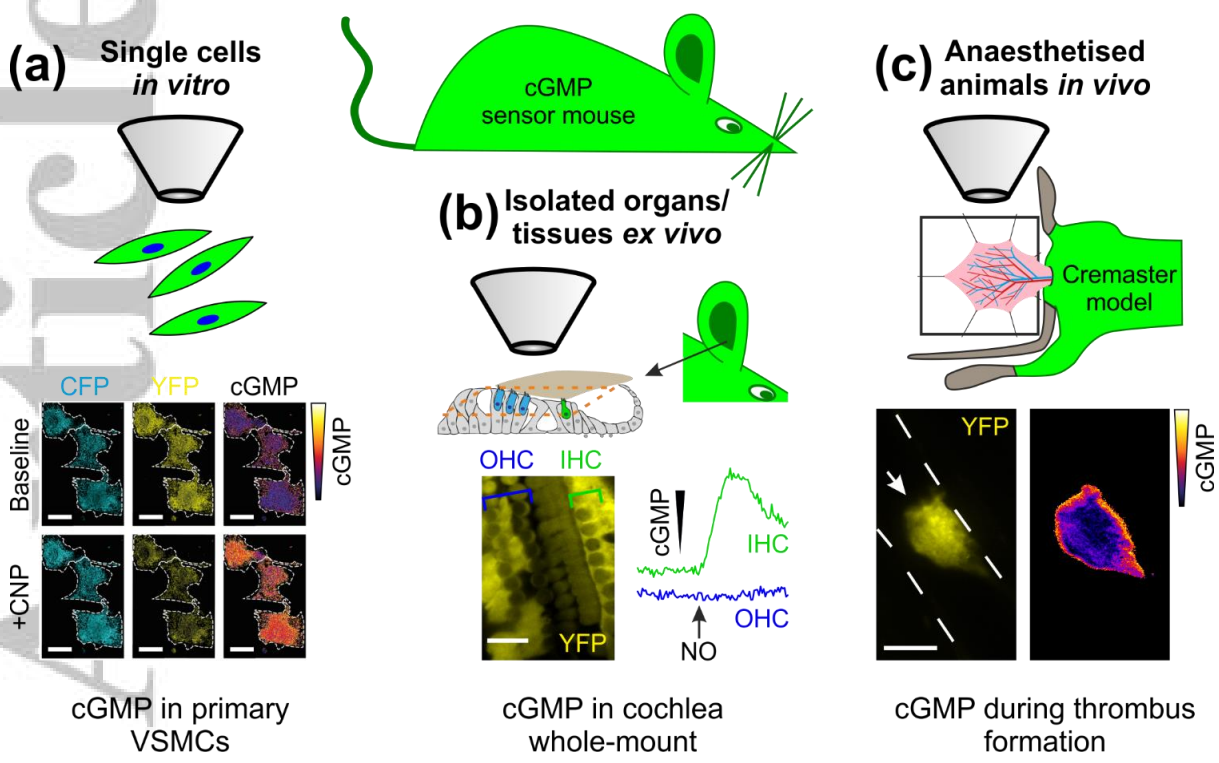


Figure 3. Applications of cGMP sensor mice for cGMP imaging. Mice expressing the cGMP sensor cGi-500 (green) globally or in distinct cell types (see section 2.2.) are a suitable basis for several imaging scenarios as illustrated in the following examples. **(a)** Imaging of single cells *in vitro*. Primary aortic VSMCs were isolated from a smooth muscle-specific cGMP sensor mouse and subjected to cGMP imaging with an epifluorescence microscope. Compared to baseline conditions, superfusion of the cells with CNP (50 nM) led to an increase of the intracellular cGMP concentration as indicated by the CFP/YFP emission ratio (cGMP, right panels). Cell borders are indicated by dashed lines. **(b)** Imaging of isolated organs and tissues *ex vivo*. A cochlea whole-mount sample was isolated from a global cGMP sensor mouse and analysed via confocal spinning disk microscopy. YFP fluorescence confirmed expression of cGi-500 in the cochlea (lower left). Note that differences in YFP intensity in different regions of the cochlea are due to heterogenous sensor expression and do not reflect different cGMP levels in these regions. Evaluation of the CFP/YFP ratio (lower right) revealed that inner hair cells (IHCs, green ratio trace) can generate cGMP in response to NO, whereas outer hair cells (OHCs, blue ratio trace) cannot. **(c)** Imaging of mice *in vivo*. The cremaster muscle of an anaesthetised platelet-specific cGMP sensor mouse was dissected. Platelet cGMP was monitored with a spinning disk microscope in real time in a blood vessel of the living animal during mechanically induced thrombus formation. YFP fluorescence (lower left) confirmed sensor expression. Analysis of the CFP/YFP ratio (lower right) revealed spatial differences of the intraplatelet cGMP concentration consistent with mechanosensitive cGMP signalling, *i.e.* the outer regions of the thrombus, which are directly exposed to fluid shear stress, appeared to have higher cGMP levels than the thrombus core. The vessel wall is indicated by dashed lines. The white arrow represents the direction of blood flow. All scale bars are 20 µm. The images shown in panel (a), (b), and (c) were taken from Thunemann et al. (2013b), Möhrle et al. (2017), and Wen et al. (2018), respectively, with permission of the respective publishers.

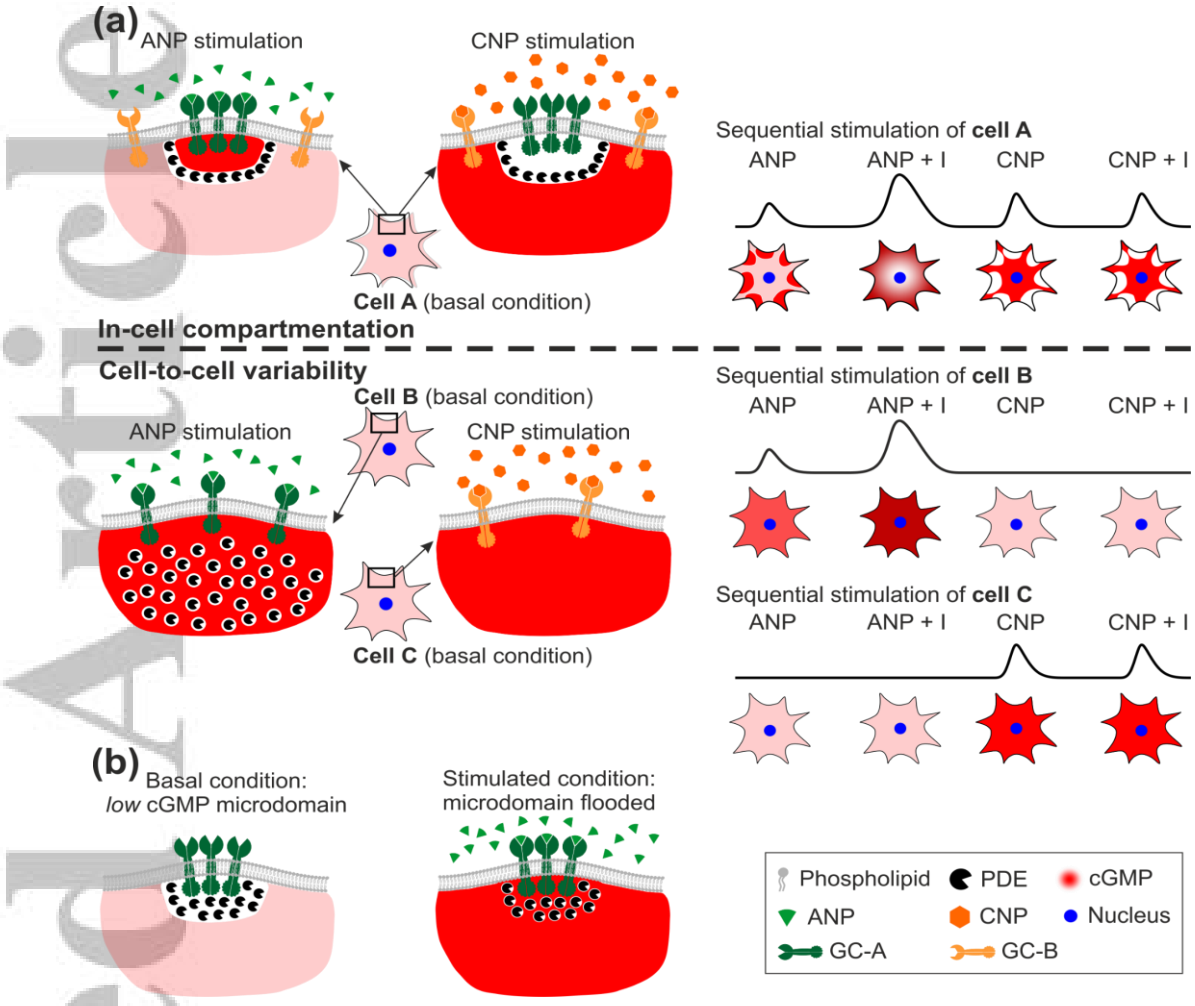
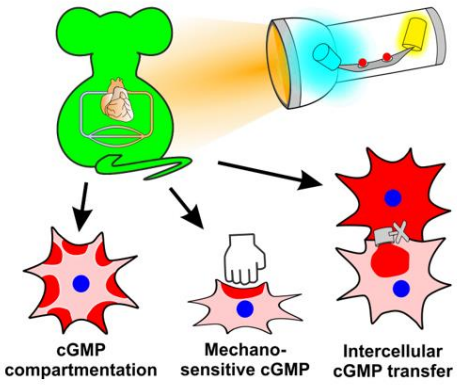


Figure 4. Heterogeneity of cGMP signalling *within* and *between* cells. The concepts are illustrated by hypothetical cGMP responses (from light to deep red) to ANP and CNP in the absence and presence of a PDE inhibitor. **(a)** In-cell compartmentation [upper part of (a)]. The same cell (cell A) expresses GC-A, GC-B and a PDE that degrades preferentially GC-A- over GC-B-derived cGMP. Thus, ANP stimulation leads to a local accumulation of cGMP in the submembrane region, termed *high* cGMP microdomain (left cell section), whereas CNP stimulation leads to a widespread cGMP increase, termed global cGMP signal (right cell section). In a cGMP imaging experiment (cGMP trace), sequential stimulation of cell A with ANP, ANP + PDE inhibitor (I), CNP, and CNP + I would show that both ANP and CNP increase the cGMP level, but only the ANP-induced cGMP signal is potentiated by the PDE inhibitor. Cell-to-cell variability [lower part of (a)]. The expression profile of cells in the analysed population differs, with cell B expressing GC-A and PDE (left cell section) and cell C only GC-B but not PDE (right cell section). Thus, cGMP imaging upon sequential stimulation would reveal the following results (cGMP traces): cell B responds to ANP and the PDE inhibitor, but not to CNP, while the cGMP level of cell C is elevated by CNP but not ANP or PDE inhibitor. Note that it is difficult to address the issue of cGMP compartmentation *vs.* cell-to-cell heterogeneity in experiments, in which not the *same* cell has been stimulated with all agents of interest, in our example, ANP, ANP + I, CNP, and CNP + I. **(b)** The new model of *low* cGMP microdomains. Recently, the model of *high* cGMP compartments in stimulated cells [as shown in (a), top left cell section] has been challenged. Instead of PDEs preventing agonist-stimulated cGMP from spreading across the cell, local accumulation of PDEs creates subcellular regions, where the free cGMP concentration is below that found throughout most of the cell under *basal* conditions (left cell section). Upon stimulation of guanylyl cyclases, the low cGMP compartments are flooded together with the entire cell (right cell section). The symbols are explained in the box below right. For further explanation, see main text.

Shining light on cGMP signalling



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