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Roland Seifert *Editor*

Non-canonical Cyclic Nucleotides

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Preface

In general, the purpose of the series *Handbook of Experimental Pharmacology* is to critically review and summarize the state-of-the-art of a well-established scientific field. For this volume, this situation is quite different: Here, experts from various disciplines discuss a young field. The development of the area was impeded by previous methodological difficulties that have been overcome only recently. Accordingly, the book also discusses scientific gaps in our knowledge and aims to foster further research. I am thankful to the Editors of the *Handbook of Experimental Pharmacology* for giving me the opportunity to put together this book.

The book is about cyclic nucleotides (cNMPs). cAMP and cGMP are firmly established second messenger molecules with broad pharmacotherapeutic implications. These two cNMPs are also designated as ***canonical cNMPs***. cAMP and cGMP have several relatives that are designated as ***noncanonical cNMPs***. A major focus of this book is to discuss the cyclic pyrimidine nucleotides cCMP and cUMP with respect to their endogenous and bacterial generators, effectors, biological functions, and elimination. The chapters cover both conceptual and methodological aspects. Most importantly, the recent development of the field was strongly enhanced by new analytical methods for cNMP detection and the synthesis of cNMP analogs that can be used as experimental tools.

In addition to cCMP and cUMP, this book covers cNMPs related to cGMP, i.e., cIMP, 8-nitro-cGMP, and the cyclic dinucleotide cGAMP. Hence, this volume is a logical continuation of Volume 191 of the *Handbook of Experimental Pharmacology* (2009) edited by H.H. Schmidt, F. Hofmann, and J.P. Stasch, entitled *cGMP: Generators, Effectors and Therapeutic Implications*. Lastly, the present volume covers 2′,3′-cNMPs. The major difference between 2′,3′-cNMPs and the cNMPs mentioned above (cAMP, cGMP, cCMP, cUMP, cIMP, and 8-nitro-cGMP) is the nature of the phosphodiester bond (3′,5′ in the latter compounds). However, this apparently small chemical difference has major consequences for biological effects.

As is expected from a young field, currently, there is not yet an established pharmacotherapeutic application. More systematic basic research in multiple biological systems is required. Nonetheless, it is beginning to emerge that each cNMP plays a unique functional role that could be exploited therapeutically. One particularly prominent case is the cUMP generated by *Pseudomonas aeruginosa*

toxin ExoY. Targeting cUMP may be an approach to treat infection with this Gram-negative bacterium, a major problem in hospitalized patients and patients with cystic fibrosis.

I am very indebted to all the authors who prepared their chapters so carefully and implemented all my suggestions for revision. All authors of this book have made substantial experimental, methodological, and/or conceptual contributions to the field and present personal but balanced views on specific aspects.

I also would like to thank the editorial team from Springer for their dedication to complete publication of the book.

As with many multiauthor books, it took more time to complete all chapters than originally planned, a major reason being that several authors wished to include very recently published peer-reviewed data into their chapters. After completion of several chapters, some new and important findings were published. Specifically, the natural compound thymoquinone was identified as a pharmacological tool to manipulate cIMP levels [Detremmerie et al. (2016) J Pharmacol Exp Ther 358:558]. Moreover, crystal structures of HCN channel 2 in complex with non-canonical cNMPs have been resolved [Ng et al. (2016) Structure 24:1629]. These data highlight that even in a small field, scientific progress can be so fast that it is difficult for a multiauthor book to be at the cutting edge.

I hope that this book offers a suitable platform for both cNMP aficionados and newcomers to conduct productive research in this exciting area. If the field develops, perhaps, in 20 years, another volume on the topic is warranted in the *Handbook of Experimental Pharmacology*.

Hannover, Germany Roland Seifert
September 2016

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cCMP and cUMP Across the Tree of Life: From cCMP and cUMP Generators to cCMP- and cUMP-Regulated Cell Functions

Roland Seifert

Contents

Abstract

The cyclic purine nucleotides cAMP and cGMP are well-established second messenger molecules that are generated by distinct nucleotidyl cyclases (NCs) and regulate numerous cell functions via specific effector molecules. In contrast, the existence of the cyclic pyrimidine nucleotides cCMP and cUMP has been controversial for many years. The development of highly specific and sensitive mass spectrometry methods has enabled the unequivocal detection and quantitation of cCMP and cUMP in biological systems. These cNMPs occur broadly in numerous mammalian cell lines and primary cells. cCMP has also been detected in mouse organs, and both cCMP and cUMP occur in various developmental stages of the zebrafish *Danio rerio*. So far, the soluble guanylyl cyclase (sGC) and soluble adenylyl cyclase (sAC) have been identified as cCMP and cUMP generators. Dissociations in the expression patterns of sAC and sGC relative to

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cCMP and cUMP abundance may point to the existence of hitherto unidentified cCMP- and cUMP-generating NCs. The broad occurrence of cCMP and cUMP in vertebrates and the distinct cNMP patterns suggest specific roles of these cNMPs in the regulation of numerous cell functions.

Keywords
Biological systems • cCMP • cUMP • Soluble adenylyl cyclase • Soluble guanylyl cyclase

1 Introduction

The cyclic purine nucleotides cAMP and cGMP are well-established second messenger molecules and are also referred to as canonical cyclic nucleotides (cNMPs) (Seifert et al. 2015) (Fig. 1a, b). In order to qualify as second messenger, a molecule must fulfill the following four criteria: (1) Generation by a first messenger-regulated enzyme; (2) activation of specific effector proteins; (3) defined biological functions; (4) specific inactivation mechanisms (Seifert 2015; Seifert et al. 2015). Several second messengers fulfill two additional criteria: (a) Mimicry by membrane-permeable second messenger analogs; and (b) mimicry by bacterial toxins (Seifert 2015; Seifert et al. 2015). In some cases, membrane-permeable second messenger analogs are not available and/or they fail to mimic the natural spatiotemporal distribution (compartmentalization). Along the same line, not all second messengers have a toxin mimicry and/or the toxin does not reflect the natural second messenger compartmentalization. cAMP and cGMP fulfill all of the six second messenger criteria introduced above (Seifert 2015; Seifert et al. 2015).

The cyclic pyrimidine nucleotides cCMP and cUMP belong to the class of noncanonical cNMPs and fulfill most of the criteria of second messengers (Seifert 2015; Seifert et al. 2015). Table 1 summarizes the current knowledge on cCMP and cUMP with regard to the established second messenger criteria. Figure 1c provides an overview of our current knowledge of cCMP signaling and Fig. 1d provides an overview on cUMP signaling. This figure also allows a side-by-side comparison of the similarities and dissimilarities with cAMP and cGMP.

In contrast to cAMP and cGMP, the existence of cCMP and cUMP has been controversial for many years (Seifert 2015; Seifert et al. 2015). While for cAMP and cGMP, sensitive and specific immunological methods, fluorescence sensor methods and radiometric methods have been developed (Post et al. 2000; Bähre and Kaever 2017), no reliable fluorescence sensor and immunological methods for cCMP and cUMP detection are available. Previous mass-spectrometric methods for cCMP and cUMP detection lacked sensitivity and specificity (Newton et al. 1984), resulting in erroneous data regarding the occurrence of these cNMPs in biological systems (Seifert 2015; Seifert et al. 2015).

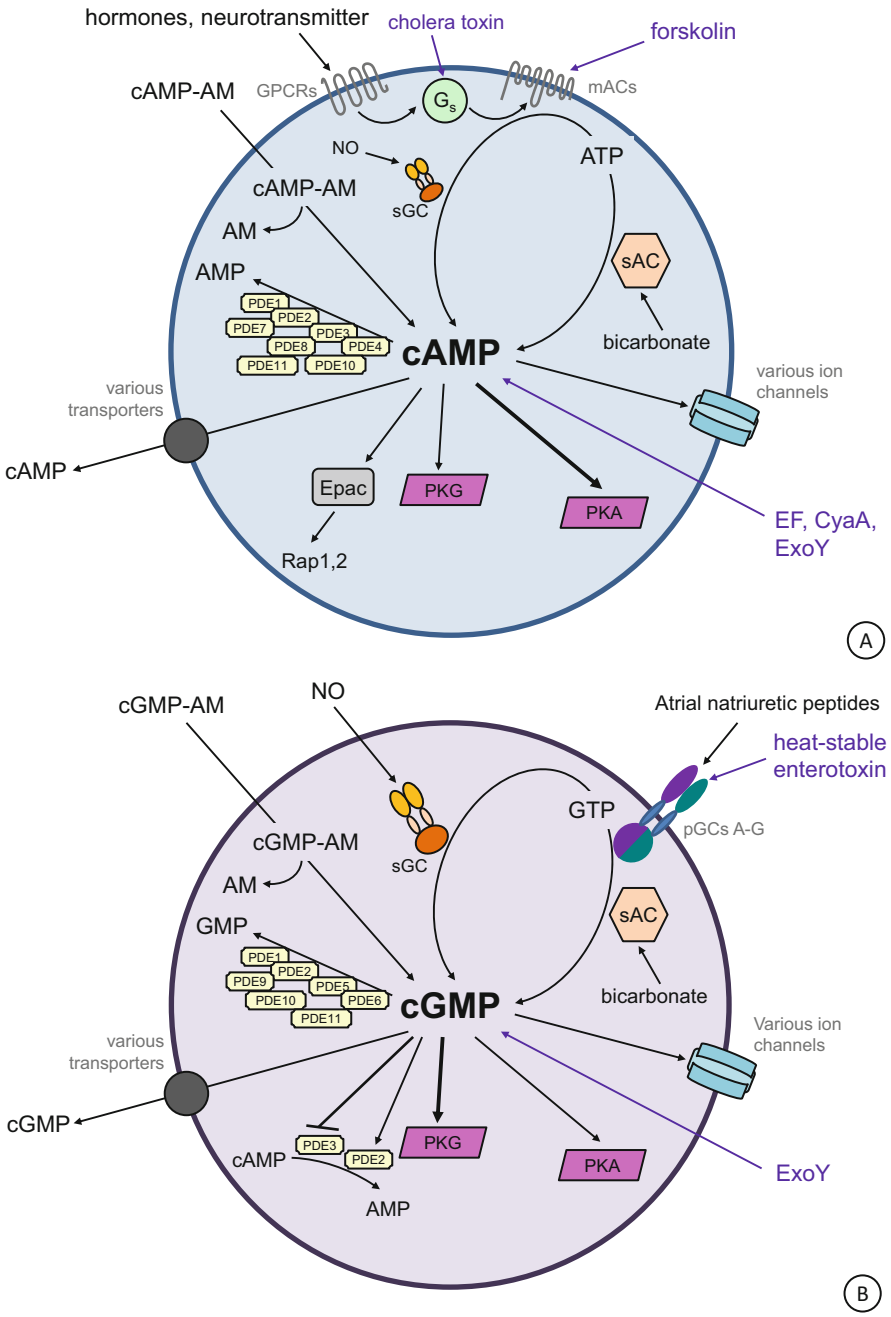

Fig. 1 Overview of cNMP-mediated signal transduction in mammalian cells. cAMP and cGMP are classic second messengers, also referred to as canonical cNMPs. (**a**) cAMP-mediated signaling; (**b**) cGMP-mediated signaling. cAMP and cGMP are generated by distinct nucleotidyl cyclases (NCs) and activate specific protein kinases, but both at the level of generation and degradation, there is some cross-talk between cAMP and cGMP. Cholera toxin ADP-ribosylates

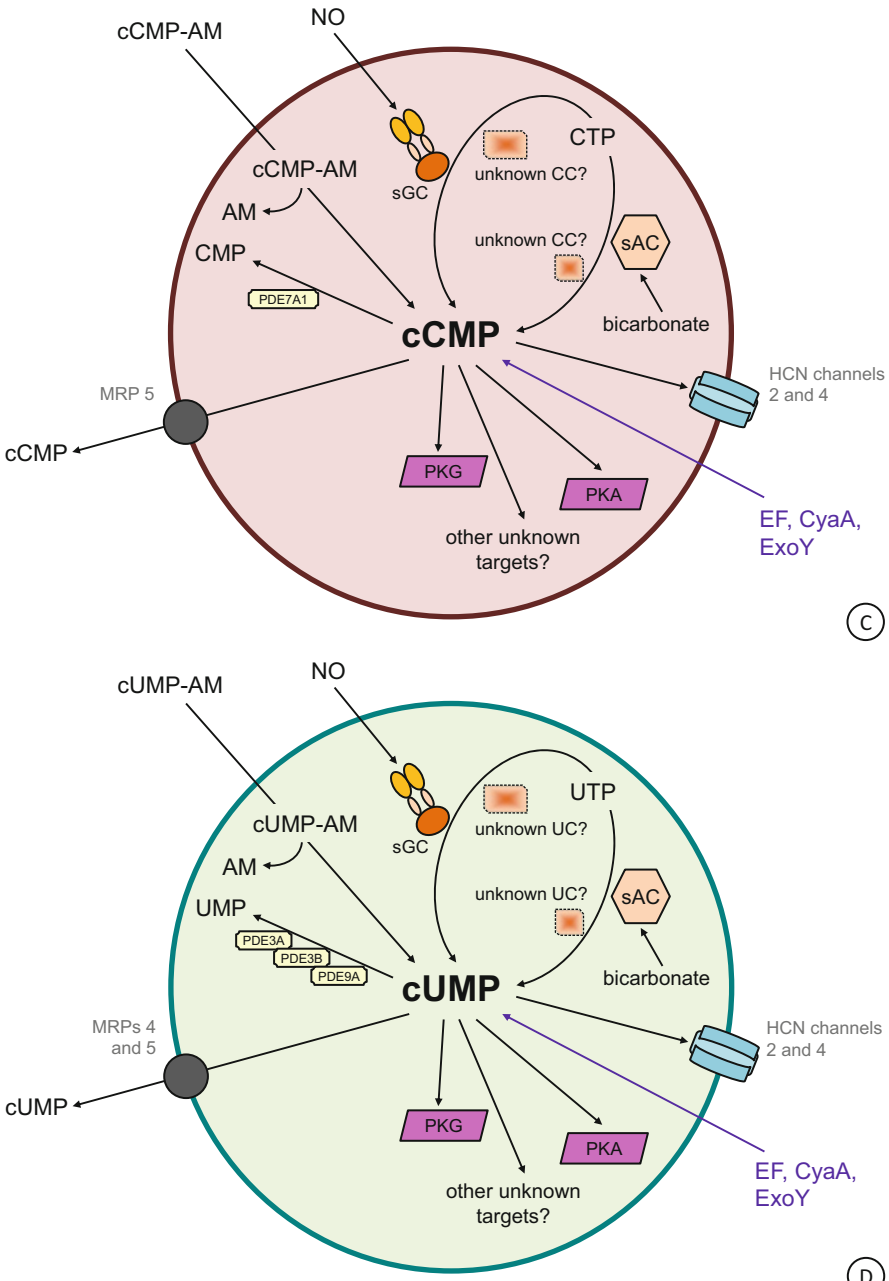

Fig. 1 (continued) and permanently activates the stimulatory G-protein G_s of membranous ACs (mACs). The plant diterpene forskolin directly activates membranous ACs (mACs), while EF, CyaA, and ExoY increase cytosolic cAMP levels. cAMP and cGMP are degraded by distinct PDEs and can also be exported into the extracellular space by different transporters. cGMP also

The recent development of modern HPLC-MS/MS methods for cNMP quantitation (Bähre and Kaever 2017) and HPLC-MS/TOF methods for unequivocal cNMP identification (Bähre et al. 2015) has overcome at least part of these serious methodological problems in cCMP and cUMP research. The chapter by Bähre and Kaever (2017) discusses methods for cCMP and cUMP quantitation. The present chapter focuses on the occurrence of cCMP and cUMP in biological systems and cCMP and cUMP generators, taking advantage of the recently developed HPLC-MS/MS and HPLC-MS/TOF methods. Based on the occurrence of cCMP and cUMP, possible biological functions of these molecules will be discussed as well.

The chapter by Dove (2017) focuses on the structural aspects of adenylyl cyclase (AC) regulation and function. Lorenz et al. (2017) cover cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) as cCMP and cUMP effector, and the exchange factor activated by cAMP (Epac) is covered by Rehmann (2017). Van Schouven and Melacini (2017) discusses hyperpolarization-activated cNMP-gated (HCN) channels as effectors for cCMP and cUMP. Schlossmann and Wolfertstetter (2017) discuss biochemical strategies for the identification of cNMP effector systems. Schneider and Seifert (2017) deal with cNMP elimination by phosphodiesterases (PDEs) and cNMP export via multidrug resistance proteins (MRPs). Grundmann and Kostenis (2017) discuss holistic approaches to dissect cCMP and cUMP effector pathways. Schwede et al. (2017) present the synthesis of membrane-permeable cCMP and cUMP analogs as pharmacological tools to elicit biological effects.

Fig. 1 (continued) regulates, in an inhibitory or stimulatory manner, the activity of cAMP-degrading PDEs. (**c**) cCMP-mediated signaling; (**d**) cUMP-mediated signaling. To this end, soluble guanylyl cyclase (sGC) and soluble adenylyl cyclase (sAC) are the only known mammalian NCs that generate cCMP and cUMP. Indirect evidence points to the existence of other as yet unidentified cCMP- and cUMP-generating NCs (CC for cCMP and UC for cUMP). cCMP and cUMP exert their effects via known cAMP/cGMP effectors (protein kinase A (PKA), protein kinase G (PKG) and HCN channels 2 and 4), but there may be other as yet unknown cCMP/cUMP target proteins. cCMP and cUMP are differentially degraded by phosphodiesterases (PDEs) and differentially exported via multidrug resistance proteins (MRPs). As experimental tools to elucidate the functions of individual cNMPs, the cNMP-acetoxymethylesters are very valuable. The cNMP-AMs can be synthesized for any cNMP (unlike the dibutyryl cNMP derivatives). cNMP-AMs are lipophilic and cross the plasma membrane. Intracellularly, cNMP-AMs are hydrolyzed to yield the free cNMP. Not shown in this figure is the possibility that cNMPs themselves are taken up from the extracellular space via transport mechanisms and that extracellular cNMPs are metabolized to biologically active degradation products

Table 1 Overview of cCMP and cUMP as second messengers

Parameter	cCMP	cUMP
Generators	NO-stimulated sGC; bicarbonate-stimulated sAC; other as yet unidentified NCs? Neither mACs nor pGCs	NO-stimulated sGC; bicarbonate-stimulated sAC; other as yet unidentified NCs? Neither mACs nor pGCs
Effectors	PKA; PKG; HCN channels 2 and 4; other as yet unidentified NCs?	PKA; PKG; HCN channels 2 and 4; other as yet unidentified NCs?
Biological functions	Apoptosis, vasodilation. Overall, very little is known about the function of cCMP, but its widespread occurrence in primary cells, cultured cells, and organs points to numerous potential functions	Apoptosis. Overall, very little is known about the function of cUMP, but its widespread occurrence in primary cells, cultured cells, and organs points to numerous potential functions. Particularly the massive cUMP increase in lung and serum following ExoY exposure points to a pathophysiological role of cUMP in lung damage and sepsis
Inactivation	PDE7A1 (degradation); MRP5 (export)	PDEs 3A, 3B, and 9A (degradation); MRPs 4 and 5 (export)
Membrane-permeable analogs	cCMP-AM (allows comparison with cUMP-AM); Dibutyryl-cCMP (no comparison possible with cUMP due to chemical reasons). Cautionary note: CMP-AM does not necessarily mimic physiological cCMP compartmentalization	cUMP-AM (allows comparison with cCMP-AM). Cautionary note: cUMP-AM does not necessarily mimic physiological cUMP compartmentalization
Bacterial toxins	CyaA, EF, and ExoY	CyaA, EF, and ExoY; studies in intact mice show that in vivo, ExoY predominantly produces cUMP
Some important open questions	Do specific mammalian cytidylyl cyclase and specific cCMP effector systems exist? (Patho)physiological importance of cCMP still largely elusive.	Do specific mammalian uridylyl cyclase and specific cUMP effector systems exist? (Patho)physiological importance of cUMP still largely elusive, but ExoY important tool to selectively increase cUMP in vivo

cCMP and cUMP fulfill many criteria of second messengers, but there are still may important gaps in our knowledge. It will require the effort from numerous laboratories with different expertise to fill these important gaps. Clearly, much of the required research possesses the character of a fishing expedition and entails high risk-taking

2 Overview of cAMP and cGMP Signaling

cAMP is generated by membranous adenylyl cyclases (mACs) that are under the control of G-protein-coupled receptors via regulatory heterotrimeric G-proteins (Seifert et al. 2015). cAMP is also generated by the bicarbonate-stimulated sAC that serves the role as metabolic sensor (Steegborn 2014). cAMP activates intracellular effector molecules including PKA, HCN channels, and Epac (Seifert et al.

2015). cAMP regulates virtually every cell function ranging from neurotransmission, immune cell function, endocrine and metabolic processes to cardiovascular function (Seifert et al. 2015). cAMP is inactivated by specific PDEs and via export through MRPs (Seifert et al. 2015). Figure 1a provides an overview of cAMP signaling.

cGMP is generated by the nitric oxide (NO)-stimulated sGC and atrial natriuretic peptide-stimulated particulate guanylyl cyclases (pGCs) (Friebe et al. 2015). By analogy to the cAMP-PKA system, cGMP activates PKG and specific HCN channels (Friebe et al. 2015). cGMP inactivation proceeds via specific PDEs and MRPs (Seifert et al. 2015). Like cAMP, cGMP regulates numerous cell functions (Friebe et al. 2015; Seifert et al. 2015). Figure 1b presents an overview on cGMP signaling. An entire volume on the cGMP generators, cGMP effectors, cGMP-regulated cell functions, and cGMP inactivation was published in the Handbook of Experimental Pharmacology some years ago (Schmidt et al. 2009). Several chapters in the present volume, mentioned in the preceding paragraph, cover several some aspects of cGMP generators, effectors, and function. It is also important to mention that there is a substantial degree of promiscuity between cAMP and cGMP signaling with regard to generators and effectors. This topic is covered in the chapters by Dove (2017), Lorenz et al. (2017), and Schlossmann and Wolfertstetter (2017).

3 cCMP and cUMP Generators

3.1 Initial Problems and Revitalization of the Field

In the 1970s, research on ACs was flourishing (Sutherland 1970), and research on GCs was ramping up as well (Goldberg et al. 1973). At that time, AC and GC research largely hinged on the use of radiometric methods with cell-free preparations. These methods took advantage of the conversion of [α-^{32}P]NTP to the corresponding [^{32}P]cNMP and subsequent separation of substrate and product by alumina-based column chromatography (Post et al. 2000). Although the radiometric NC method is, in principle, reliable and robust (Post et al. 2000), nonetheless, the method produced artifacts when it was applied to the tentative identification of cytidylyl cyclase (CC) activity in mammalian tissue homogenates (Cech and Ignarro 1977; Gaion and Krishna 1979). Probably, the column matrix was clogged with large amounts of protein. Consequently, the substrate [α-^{32}P]CTP was not retained by the alumina but eluted and erroneously assumed to be [^{32}P]cCMP. However, when applied properly, the classic alumina column method is well suited to detect CC activity as exemplified for the CC activity of certain bacterial toxins (Göttle et al. 2010). The unfortunate high-profile publication on a tentative mammalian CC that was never officially retracted and the confusion about the suitability of the classic radiometric assay for CC analysis substantially impeded development of cCMP (and cUMP) research, and skepticism in the research community was large so that only very few groups were actively pursuing research in this field. Early mass-spectrometric methods were also not sufficiently

sensitive and specific to revive the field and provide convincing evidence for the existence of a specific CC in mammalian systems (Newton et al. 1984).

The dormant field was revitalized serendipitously during the course of pharmacological studies that aimed at the identification of AC and GC inhibitors. It these studies, several purine and pyrimidine nucleotides were assessed for their inhibitory effects on NCs. Among the nucleotides studied was the metabolically stable UTP analog uridine 5′-[γ-thio]triphosphate (UTPγS). UTPγS inhibited sGC conventionally purified from bovine lung with a K_i value of 4 μM (Gille et al. 2004). Based on these rather surprising results the question arose whether sGC may exhibit uridylyl cyclase (UC) activity. Using a radiometric method with [α-^{32}P]UTP as substrate and alumina column separation of product and substrate, in fact, a NO-stimulated tentative UC activity of sGC was identified (Gille et al. 2004). However, formal proof of the chemical identity of the eluted compound as authentic [^{32}P]cUMP could not be obtained with this method.

Within these pharmacological studies, 2′,3′-*O*-methylanthraniloyl (MANT)-substituted UTP and CTP were found to be quite potent inhibitors of the bacterial AC toxins CyaA from *Bordetella pertussis* and edema factor from *Bacillus anthracis* (Göttle et al. 2007; Taha et al. 2009). Thus, by analogy to the situation with GC, the question arose whether the toxins exhibit CC- and UC activity. Since both toxins possess exceedingly high catalytic activity and were available in highly purified form, it was possible to validate the radiometric NC assay for UC and CC activities (Göttle et al. 2010). Complemented with HPLC and HPLC/MS-based methods, it was unequivocally demonstrated that, indeed, both CyaA and edema factor possess both UC- and CC activity (Beste et al. 2013). Moreover, ExoY from *Pseudomonas aeruginosa* turned out to be very effective at producing cCMP and particularly cUMP in native cells, cultured cell lines and in the intact organism (Beckert et al. 2014a; Bähre et al. 2015; Morrow et al. 2015). Purified ExoY, when reconstituted with cytosolic extract from various cells (containing an endogenous activator) can generate cCMP and cUMP as well (Roland Seifert, unpublished results). Detailed discussions of the historically and conceptually very important CC- and UC activities of bacterial toxins are presented in the chapter by Morrow et al. (2017). Considering then that in many cases, bacterial toxins exploit mammalian signal transduction pathways (Simon et al. 2014), the search for mammalian NCs with CC- and UC activities began again. Since mACs and pGCs turned out to be devoid of such activities (Beste et al. 2013; Hasan et al. 2014), sGC and sAC remained the only two mammalian candidate NCs among hitherto known cNMP-producing enzymes (Beste et al. 2012; Hasan et al. 2014).

3.2 Soluble Guanylyl Cyclase

In order to unequivocally demonstrate CC and UC activity of sGC, it was important to use a highly purified enzyme and a HPLC-MS/MS-based detection method. For the studies, recombinant sGC isoenzyme $\alpha_1\beta_1$ was examined (Beste et al. 2012). This enzyme was also used for the development of the NO-independent sGC

activators (Stasch et al. 2002). With GTP as cognate sGC substrate it was shown that both the radiometric NC assay and the HPLC-MS/MS-based assay yielded comparable results (Beste et al. 2012). sGC possesses both CC and UC activity, but only in the presence of Mn^{2+} and not in the presence of Mg^{2+} (Beste et al. 2012). The V_{max} value of the CC activity of sGC amounted to only 4.6% of the GC activity, and the UC activity of sGC amounted to 18.8% (Beste et al. 2012). The structural basis for these rather low NC activities of sGC is discussed by Dove (2017). In light of the low CC- and UC activities and the assumption that under physiological conditions, Mg^{2+}, but not Mn^{2+} is the metal cofactor for sGC, it seemed to be rather unlikely that in intact cells a CC- and UC activity of sGC could be observed.

In intact HEK293 cells transiently overexpressing sGC-$\alpha_1\beta_1$, a rapid and massive NO-stimulated cGMP increase was observed, followed by a late and pronounced cGMP decrease (Bähre et al. 2014). In contrast, NO-stimulated cCMP accumulation was ~3000-fold lower than cGMP accumulation and delayed in onset and a late decline (Bähre et al. 2014). NO-stimulated cUMP accumulation was ~600-fold lower than cGMP accumulation and remained at a plateau even at late time points (Bähre et al. 2014). It could be argued that the small and late cCMP and cUMP accumulations observed in intact cells overexpressing sGC-$\alpha_1\beta_1$ simply represent GTP substrate depletion for sGC with no physiological relevance. However, at the minimum, these data suggest that Mn^{2+} can serve as metal cofactor for sGC in intact cells. In future studies it will be necessary to determine the physiological Mn^{2+} concentrations in cells and to manipulate intracellular Mn^{2+} concentrations concomitant with determinations of cCMP and cUMP concentrations.

As a system with greater physiological relevance than transiently transfected HEK293 cells, RFL-6 rat fibroblasts endogenously expressing sGC were studied. The data obtained with this system were quite surprising: In contrast to the data obtained with HEK293 cells, the NO-stimulated increases in cGMP, cCMP and cUMP in RFL-6 cells were comparable (Bähre et al. 2014). These data lend support to the assumption that cCMP and cUMP represent physiologically relevant sGC products. The strikingly different results obtained with HEK293 cells and RFL6 cells are not easy to explain. It is possible that differences in elimination (both degradation by PDEs and export via MRPs) between the two cell types contribute to the distinct cNMP patterns. In addition, it must be emphasized that the HPLC-MS/MS technology is only capable of picking up average cNMP concentrations among a large population of cells, but the method cannot provide insights into the spatio-temporal cNMP distribution within a given cell. Moreover, it is possible that different substrate availability (ATP, GTP, CTP, and UTP) in cells contributes to cell type-specific cNMP responses. To test this hypothesis, cellular NTP concentrations need to be determined. But this is not trivial because NTPs are rapidly degraded to NDPs (Ipata and Balestri 2013). As another cautionary note, so far, no other systems have been identified in which NO increases cCMP and cUMP. Thus, it is premature to conclude that sGC plays a universal role in regulating cellular cCMP and cUMP levels.

3.3 Soluble Adenylyl Cyclase

In several cell types, basal cCMP and cUMP concentrations, i.e. in the absence of NO stimulation, are quite high (Hartwig et al. 2014). In addition, several mouse and rat organs contain substantial cCMP concentrations (Jia et al. 2014; Bähre et al. 2015). Furthermore, in the absence of NO, purified sGC possesses only very low catalytic activity (Beste et al. 2012). These data are not readily compatible with the assumption that sGC accounts for high basal cCMP and cUMP concentrations. Another argument against sGC playing a general role in maintaining high basal cCMP and cUMP concentrations comes from the fact that organs with high cCMP concentrations, i.e. pancreas and spleen, exhibit only low sGC expression levels (Bähre et al. 2015).

Untransfected HEK293 cells, B103 neuroblastoma and primary astrocytes exhibit particularly high cCMP and cUMP concentrations (Hartwig et al. 2014; Hasan et al. 2014). Intriguingly, HEK293 cells, having a neuronal origin (Shaw et al. 2002) express sAC (Geng et al. 2005), and astrocytes express sAC as well (Choi et al. 2012). In fact, in untransfected HEK293 cells, NO-containing compounds did not increase cCMP and cUMP concentrations. Instead, the addition of $NaHCO_3$ robustly increased cCMP and cUMP concentrations, whereas the removal of $NaHCO_3$ decreased cCMP and cUMP concentrations (Hasan et al. 2014). Along the same line, the sAC inhibitor 2-(1H-benzo[d]imidazole-2-ylthio)-*N*′-(5-bromo-2-hydroxybenzylidene)propanehydrazide (KH7) reduced basal cCMP and cUMP concentrations in untransfected HEK293 cells (Hasan et al. 2014). Similar data were obtained with B103 neuroblastoma cells. As a cautionary note, KH7 is, unfortunately, not a specific sAC inhibitor and newer more specific sAC inhibitors have not yet been studied in the context of cCMP and cUMP (Ramos-Espiritu et al. 2016). Additionally and in marked contrast to sGC (Beste et al. 2012), an extensive biochemical characterization of the substrate-specificity of purified sAC beyond GTP (Kleinboelting et al. 2014) is not yet available. Preliminary data show that CTP and UTP are sAC substrates (Dove 2017). Despite these shortcomings, the available data suggest that at least in certain cell types, sAC plays a role in maintaining and regulating basal cCMP and cUMP concentrations.

However, it is also clear that sAC cannot account for basal cCMP and cUMP concentrations in all biological systems. This assumption is supported by four major findings: First, mouse testis expresses very high sAC levels, but basal cCMP levels are exceedingly low, and cUMP is not detected at all (Bähre et al. 2015). Conversely, pancreas and spleen contain quite high cCMP concentrations, but sAC expression levels are very low (Bähre et al. 2015). Second, in several developmental stages and organs from the zebrafish *Danio rerio*, cCMP and cUMP are clearly detected, but this organism does not express sAC (Dittmar et al. 2015). Third, HEK cells transiently or stably overexpressing sAC did not show increased cellular cCMP and cUMP levels (Sabine Wolter and Roland Seifert, unpublished results). Fourth, CTP and UTP are only poor sAC substrates (Dove 2017). At the time being, there is no direct evidence for the existence of a specific mammalian CC

or UC. However, it cannot be excluded that the human genome contains genes encoding for hitherto unknown NCs that are structurally different from the known enzymes. In fact, the human genome still contains many unknown genes involved in signal transduction (Mikolcevic et al. 2012; Ngo et al. 2016).

4 Occurrence of cCMP and cUMP in Biological Systems

Using the highly sensitive and specific HPLC-MS/MS method described by Bähre and Kaever (2017), numerous types of cultured cell lines, primary cells and organs were assessed with respect to the occurrence of cCMP and cUMP under basal conditions (Hartwig et al. 2014). It should be kept in mind that the exact metabolic and functional status of the systems was not precisely defined, but this could have an impact on the actual cNMP concentrations (Choi et al. 2012; Hasan et al. 2014).

Table 2 provides an overview on cNMP concentrations in selected mammalian cell culture lines. In all six species examined so far (human, monkey (primates), dog (carnivore), rat, mouse, and hamster (rodents)), cCMP and cUMP were detected. Thus, it can be safely extrapolated that all mammalian species possess cCMP and cUMP. In general, cAMP is the most abundant cNMP, but the total concentrations vary by more than 500-fold. It is currently unknown to which extent different NC activities, PDE activities and transporter activities contribute to these striking differences in basal cNMP patterns among different cells. In general, the concentrations of cGMP, cCMP, and cUMP are lower than cAMP concentrations. cCMP and cUMP are detected in cell lines of epithelial, mesenchymal, and neuronal origin. In some cell lines the cCMP and cUMP concentrations surpass the cGMP concentrations. Overall, any given cell type exhibits its unique cNMP pattern, pointing to the existence of a hitherto unknown cNMP signaling code (Seifert 2015; Seifert et al. 2015). In HEK293 cells and B103 cells, the high cCMP and cUMP concentrations have been attributed to sAC (Hasan et al. 2014). The roles of cCMP and cUMP have so far only been studied in few systems (Wolter et al. 2017), highlighting the large need for functional studies.

cCMP and cUMP also occur in various types of primary cells (Table 3). Overall, similar general statements on cNMP patterns as for cultured cell lines can be made. The highest cCMP and cUMP concentrations were observed in astrocytes. In these cells, sAC plays a regulatory role in homeostasis (Choi et al. 2012), but the functional role of cCMP and cUMP has still to be defined. Particular attention should also be paid to endothelial cells. Both human and rat endothelial cells contain cCMP and cUMP (Hartwig et al. 2014; Morrow et al. 2015). In rat endothelial cells, cCMP and cUMP concentrations are much higher than in human cells (Table 3). It is not clear whether this represents a species-specific difference or reflects a difference in cell culture conditions. In the rat endothelial cells, basal cCMP and cUMP concentrations are elevated by exposure to ExoY (Morrow et al. 2015). ExoY induces profound functional and morphological changes in endothelial cells (Morrow et al. 2015), but the precise function of cCMP and cUMP in these cells has not yet been defined. One approach to

Table 2 Basal cNMP concentrations in cultured mammalian cell lines

Species	Cell line	Origin	cAMP	cGMP	cCMP	cUMP
Human	HEK293	Neuronal, despite designation as human embryonic kidney cells	160	73	33	55
Human	A549	Lung epithelial cells	29	24	3.5	11
Human	Capan1	Pancreas epithelial cell	0.6	<LLOQ	0.2	0.3
Human	HT-29	Colorectal carcinoma epithelial cell	0.9	<LLOQ	<LLOQ	<LLOQ
Human	LnCap	Prostate carcinoma	2.2	<LLOQ	0.1	0.8
Human	HeLa	Cervix adenocarcinoma cells	3.2	1.5	0.3	0.7
Human	MDA	Mammary gland epithelial cells	2.7	<LLOQ	0.2	0.4
Human	MCF-7	Mammary gland epithelial cells	4.0	<LLOQ	0.1	0.5
Human	MCF-7.41	Mammary carcinoma cells	2.6	<LLOQ	0.1	1.6
Human	Jurkat	T-lymphocytes (acute T-cell leukemia)	0.4	<LLOQ	0.1	<LLOQ
Human	MOLT-3	Lymphoblastoid cells	0.4	<LLOQ	0.8	<LLOQ
Human	PLB-985	Promyelocytic leukemia cells	0.2	<LLOQ	0.2	<LLOQ
Human	HL-60	Promyelocytic leukemia cells	11	0.3	0.1	0.3
Human	U937	Promonocytic cells	1.0	0.1	<0.1	<LLOQ
Human	K562	Erythroleukemia cells	2.1	<LLOQ	1.2	0.6
Human	HEL	Erythroleukemia cells	0.4	<LLOQ	<LLOQ	<LLOQ
Human	SK-ES1	Osteosarcoma cells	2.7	<LLOQ	0.4	1.0
Monkey	COS	Fibroblast-like kidney cells	120	52	16	25
Dog	MDCK	Kidney epithelial cells	120	10	12	31
Rat	B103	Neuroblastoma cells	83	7.5	14	22
Mouse	J774	Macrophage cells	17	1.2	0.5	1.2
Mouse	S49	Thymocyte T-lymphoma cells	5.6	<LLOQ	0.1	<LLOQ
Hamster	CHO	Ovary cells	50	10	8.0	6.7

Cells were harvested, and cNMPs were determined by HPLC-MS/MS. Data are expressed in pmol/mg protein except for human blood cells. For these cells, data are expressed in pmol/10^6 cells. LLOQ, lower limit of quantitation. Data are adapted from Hartwig et al. (2014). Numbers were rounded and data variation was eliminated to facilitate comparison of data shown in Tables 2, 3, and 4 and data from different research groups

differentiate the functions of cCMP and cUMP could be to design bacterial toxins that exclusively generate cCMP or cUMP.

In amphibian tissue (*Ambystoma mexicanum*), cCMP and cUMP have not been detected (Table 3). Further amphibian tissues and species have not yet been studied.

Table 3 Basal cNMP concentrations in primary mammalian cells, amphibians, worms, insects, fungi, amoeba, bacteria, and plants

Species	Cell type	cAMP	cGMP	cCMP	cUMP
Human	Neutrophils	0.5	0.1	<LLOQ	<LLOQ
Human	Platelets	6.0	2.1	<LLOQ	<LLOQ
Human	Monocytes	0.2	0.1	<LLOQ	<LLOQ
Human	Th2 lymphocytes	20	1.5	0.9	0.7
Human	Hepatocytes	4.3	0.2	0.2	0.4
Human	Pulpa stem cells	16	1.1	1.4	2.0
Human	Endothelial cells	4.6	0.5	0.3	1.0
Rat	Astrocytes	250	14	22	41
Rat	Microglia	59	<LLOQ	<LLOQ	<LLOQ
Rat	Cardiac fibroblasts	20	0.4	1.5	<LLOQ
Rat	Pulmonary artery endothelial cells	~80	~7	~3	~10
Rat	Pulmonary microvascular endothelial cells	~100	~8	~8	~20
Ambystoma mexicanum	Tail blastema	1.1	0.6	<LLOQ	<LLOQ
Caenorhabditis elegans	Bristol, N2	15	6.0	<LLOQ	<LLOQ
Drosophila melanogaster	Adult animals	12	2.5	<LLOQ	<LLOQ
Spodoptera frugiperda	Sf9 cells, ovary	23	<LLOQ	<LLOQ	<LLOQ
Saccharomyes cerevisiae	Diploid cells	1.9	<LLOQ	<LLOQ	<LLOQ
Dictyostelium discoideum	Strain AX2	11	4.5	<LLOQ	<LLOQ
Escherichia coli	Strain K12	55	2.0	<LLOQ	<LLOQ
Pseudomonas aeruginosa	Strain F469	0.6	<LLOQ	<LLOQ	<LLOQ
Arabidopsis thaliana	Green leaves	16	1.3	<LLOQ	5.2

Cells were harvested, and cNMPs were determined by HPLC-MS/MS. Data are expressed in pmol/mg protein except for human blood cells. For these cells, data are expressed in pmol/10^6 cells. LLOQ lower limit of quantitation. All data except for data on rat endothelial cells are adapted from Hartwig et al. (2014). Data for rat endothelial cells were adapted from Morrow et al. (2015). Numbers were rounded and data variation was eliminated to facilitate comparison of data shown in Tables 2, 3, and 4 and data from different research groups

The model plant *Arabidopsis thaliana* contains cUMP (but not cCMP) in green leaves. The analysis of the functional role of cCMP and cUMP in plants is an emerging field and being dealt with by Marondedze et al. (2017). *Caenorhabditis elegans*, *Drosophila melanogaster*, *Dictyostelium discoideum,* and *Saccharomyces cerevisiae* are well-established model organisms for signal transduction research, but in these species, neither cCMP nor cUMP was detected (Hartwig et al. 2014). *Spodoptera frugiperda* Sf9 cells are broadly used as expression systems for

mammalian proteins involved in cNMP metabolism (Schneider and Seifert 2010; Seifert et al. 2012). These cells do not contain cCMP and cUMP, providing an excellent background for expression of novel proteins involved in cCMP and cUMP metabolism. Two bacterial species (*Escherichia coli* K12 strain and *Pseudomonas aeruginosa* F469 strain) were found to be devoid of cCMP and cUMP (Hartwig et al. 2014), but it cannot be excluded that other bacteria contain cCMP and cUMP and that specific culture conditions have an impact on cNMP concentrations. Overall, these data indicate that cCMP and cUMP, in contrast to cAMP, have a considerably more restricted occurrence (and probably also function) than cAMP. But even the established second messenger cGMP is not found in all species (Table 3).

Previous studies on the occurrence of cCMP and cUMP in organs yielded erroneous results due to insufficient sensitivity and specificity of MS methods used (Newton et al. 1984; Seifert et al. 2015). In order to move towards the next level with respect to elucidation of the function of cCMP and cUMP, it was not only important to identify cCMP and cUMP in cultured cell lines (Table 2) and primary cells (Table 3), but also in intact organs (Table 4). This was quite challenging due to the fact that in intact organs, matrix effects reduce the sensitivity of HPLC-MS/MS methods. Particularly the detection of cUMP is very difficult (Bähre et al. 2015). However, despite these difficulties, positive results were obtained. As expected from the studies with cultured cell lines and primary cells, cAMP was the predominant cNMP in various rat and mouse organs, cGMP being much less abundant and even undetectable in certain organs, despite its established second messenger role. The highest cCMP concentrations were observed in pancreas, spleen, and the female reproductive system. The presence of cCMP in heart, lung, liver, spleen, and kidney was confirmed by an independent research group (Jia et al. 2014). The principal reproducibility of data is very important in a time plagued by non-reproducibility problems (Kannt and Wieland 2016) and bolsters confidence in the validity of the data. However, the results of the two groups (Jia et al. 2014; Bähre et al. 2015) cannot be directly compared in terms of quantitation because the HPLC-MS/MS methods used were not identical, different species (rat versus mouse) were used and because cNMP levels were calculated differently (pmol cNMP per g of rat tissue versus pmol cNMP per mg of mouse tissue protein). Nonetheless, these studies consistently show that cAMP is the most abundant cNMP and that roughly, cGMP and cCMP concentrations are in a similar range. In mouse organs, cUMP was not detected under basal conditions, but only in lung tissue, serum, and urine following exposure to ExoY (Bähre et al. 2015). In rat organs, cUMP was not explicitly reported (Jia et al. 2014). Perhaps, cUMP was studied but not reported as negative result.

Given the difficulties to detect cUMP in mouse organs, other species were examined, hoping to identify systems in which matrix effects impede less prominently with the detection of cUMP. In fact, the zebrafish *Danio rerio* turned out to be a feasible model organism. Similar to the data obtained for rat and mouse organs, cAMP is the predominant cNMP in zebrafish organs, and cGMP is much less abundant (Dittmar et al. 2015). Eyes, where cAMP and cGMP concentrations are similar, are a notable exception, consistent with the important role of cGMP in

Table 4 Basal cNMP concentrations in organs from mouse, rat, and zebrafish

Species	Organ	cAMP	cGMP	cCMP	cUMP
Mouse	Heart	3.3	<0.1	<0.1	<LLOQ
Mouse	Lung	4.7	0.1	0.4	<LLOQ
Mouse	Pancreas	3.7	0.5	4.8	<LLOQ
Mouse	Liver	2.8	<LLOQ	0.1	<LLOQ
Mouse	Spleen	3.3	<LLOQ	6.3	<LLOQ
Mouse	Kidney	3.1	<0.1	0.1	<LLOQ
Mouse	Bladder	5.0	<0.1	0.1	<LLOQ
Mouse	Brain	12	0.1	<0.1	<LLOQ
Mouse	Thymus	4.8	<0.1	0.4	<LLOQ
Mouse	Female reproductive system	3.3	<LLOQ	3.9	<LLOQ
Mouse	Testis	4.7	<0.1	<0.1	<LLOQ
Rat	Heart	~400	~6	~2	Not studied
Rat	Lung	~500	~5	~1	Not studied
Rat	Kidney	~400	~0	~1	Not studied
Rat	Liver	~100	~0	~1	Not studied
Rat	Spleen	~650	~6	~5	Not studied
Rat	Brain	~400	~7	~1	Not studied
Zebrafish	Embryo 24 h after fertilization	2.0	0.3	0.1	0.4
Zebrafish	Larvae 5 days after fertilization	26	1.9	0.3	0.8
Zebrafish	Eyes	3.1	3.2	<0.1	<0.1
Zebrafish	Brain	13	0.2	<0.1	<0.1
Zebrafish	Heart	19	0.4	0.4	0.7
Zebrafish	Entrails	14	0.2	<0.1	<0.1
Zebrafish	Eggs	0.4	<0.1	<0.1	<0.1
Zebrafish	Testes	14	0.6	0.1	0.5

Organs from Balb/c mice, Sprague-Dawley rats, and zebrafish were harvested, and cNMPs were determined by HPLC-MS/MS. Data are expressed in pmol/mg protein for mice and zebrafish. For rats, data are expressed in pmol/g tissue. Data for mice are adapted from Bähre et al. (2015). Data for rats are adapted from Jia et al. (2014). Data for zebrafish are adapted from Dittmar et al. (2015). Numbers were rounded and data variation was eliminated to facilitate comparison of data shown in Tables 2, 3, and 4 and data from different research groups

vision (Gross et al. 2015). Overall, cCMP and cGMP concentrations are in a similar range in zebrafish organs. Evidently, matrix effects are less relevant in the zebrafish than in rat and mouse organs because cUMP could be clearly detected in all organs studied, with the highest cUMP concentrations in the heart. There are also developmental changes in cNMP pattern in the zebrafish.

5 cCMP- and cUMP-Regulated Cell Functions

While the occurrence of cCMP and cUMP in various cell lines, primary cells, and even in organs is now well established (Tables 2, 3, and 4), it has been much more difficult to assign specific biological functions to cCMP and cUMP. There are

several reasons for this situation. Firstly, the critical tools for cCMP/cUMP research, cCMP-AM and cUMP-AM, have become available only recently (Beckert et al. 2014b; Schwede et al. 2017). Secondly, it is not clear whether cCMP and cUMP exert their biological effects in isolation or whether they act in conjunction with other cNMPs. One study suggests that at least cUMP and cGMP act in a concerted fashion (Beckert et al. 2014b). Answering this question requires systematic experiments with all possible cNMP-AM combinations in different ratios. It is also recommended that such studies include the addition of PDE and/or MRP inhibitors to delay the inactivation of the released cNMPs. Thirdly, the application of cNMP-AMs to cells may not exactly mimic the physiological situation. Specifically, cCMP and cUMP may be compartmentalized in cells, but the application of cNMP-AMs to cells probably floods the cells rather uniformly with cNMPs. Lastly, still only very few groups are working in the field, and this also entails that the number of biological systems studied so far is limited (Schneider and Seifert 2015).

Using dibutyryl-cCMP as tool, early studies suggested that cCMP plays a role in the regulation of human neutrophil superoxide production (Ervens and Seifert 1991), but follow-up studies with cCMP-AM and cUMP-AM have not yet been performed. Dibutyryl-cCMP has also been shown to induce vasodilation and inhibition of platelet aggregation via protein kinase G (Desch et al. 2010), but again, follow-up studies with cCMP-AM and cUMP-AM have yet to be performed. cCMP-AM (but not cUMP-AM) induces apoptosis in S49 lymphoma cells via a pathway that is independent of known cAMP- and cGMP effectors (Wolter et al. 2015). In HEL erythroleukemia cells, cCMP-AM and cUMP-AM differentially regulate various aspects of apoptosis (Dittmar et al. 2016). In B103 neuroblastoma cells, cUMP-AM, in combination with cGMP-AM, induces cell death, but the effect of cCMP-AM was not studied (Beckert et al. 2014b). However, even from these few and incomplete data it can be concluded that cCMP and cUMP are functionally non-equivalent. In a broader perspective, these prototype studies show that with the systematic comparison of the effects of various cNMP-AMs, valuable information about the second messenger functions of individual cNMP-AMs can be obtained. Additional (but as yet indirect) evidence for a role of cUMP in the pathogenesis of lung damage comes from studies showing that ExoY induces lung damage (Stevens et al. 2014) and rather selectively increases cUMP levels in lung, serum, and urine (Bähre et al. 2015).

Even extracellularly applied cIMP (without lipophilic AM group) can induce prominent biological effects, i.e. vasoconstriction (Detremmerie et al. 2016). The role of cIMP as second messenger is discussed by Leung et al. (2017). Either cIMP is taken up into cells via transporters or metabolized to biologically active degradation products extracellularly. In any case, these data indicate that it will also be very important to study the effects of extracellularly applied cCMP and cUMP without AM group on cell functions. For both cGMP and cAMP, extracellular effects have been observed (Seifert et al. 2015), but for cCMP and cUMP corresponding studies are largely lacking.

The studies on the occurrence of cCMP and cUMP in cultured cell lines, primary cells and organs provide hints about a possible functional role of these cNMPs. It is particularly worthwhile to study the functional role of cCMP and cUMP in astrocytes because of the high cNMP concentrations. Given the importance of astrocytes in numerous neurological and psychiatric diseases (Di Benedetto and Rupprecht 2013), studies with astrocytes are very promising. The analysis of the role of cCMP and cUMP in epithelial cells is worthwhile, too. Perhaps, cCMP and cUMP regulate secretory processes. However, even the analysis of cCMP and cUMP in cells where the endogenous concentrations of these cNMPs are low is worthwhile. Specifically, HEL cells, K562 and S49 cells possess only very low cCMP and cUMP concentrations (Table 2), but the pharmacological effects of exogenously added cNMP-AMs on apoptosis were very prominent (Wolter et al. 2015; Dittmar et al. 2016).

With respect to organs, studies on the heart are encouraged given the relatively high cUMP concentrations in zebrafish heart and the fact that heart expresses a cUMP-degrading PDE (probably PDE3) (Dittmar et al. 2016; Schneider and Seifert 2017). Here, the analysis of heart tissue in various developmental stages and in different disease situations is particularly important. In the zebrafish, the roles of cNMPs in early embryonic development can be readily studied by assessing the effects of cNMP-AMs on morphology. The presence of cCMP and cUMP in reproductive organs also warrants detailed follow-up studies in these systems. In fact, functional effects of cCMP on early embryonic development were already reported many years ago (Chan 1987), but not followed up. The high cCMP concentrations in pancreas (Bähre et al. 2015) call for functional studies on a possible role of cCMP in endocrine and exocrine function, and the presence of cCMP at high concentrations in mouse spleen highlights the necessity to conduct extensive studies on the role of cCMP in the immune system. Indeed, some studies already point to a functional role of cCMP in lymphocyte and neutrophil function (Ervens and Seifert 1991; Wolter et al. 2015). Lastly, the function of cCMP and cUMP in the brain should be studied. Early studies showed behavioral effects of intraventricularly applied cCMP in rats (Brus et al. 1984). Again, follow-up studies have not yet been performed. Although the global cCMP concentrations in brain are low and cUMP was not detected at all (Bähre et al. 2015), these data do not exclude a functional importance of these cNMPs for several reasons. First, so far only brain in its entity was studied, but not specific brain regions. Second, due to the high metabolic activity of the brain, substantial changes in cNMP concentrations may occur between sacrifice of the animals and preparation of the organ extract for HPLC-MS/MS analysis. Lastly, cCMP and cUMP may play a functional role only in specific cell types such as astrocytes (Choi et al. 2012; Hartwig et al. 2014).

6 Conclusions and Future Perspectives

After decades of dormancy and skepticism caused by published artifacts and lack of reliable analytical methods, the field of cCMP and cUMP research has made substantial progress over the past years. Mammalian enzymes and bacterial toxins

that produce cCMP and cUMP have been identified, and the same is true for effectors, PDEs, and transporters. In addition, the broad occurrence of cCMP and cUMP in mammalian systems is suggestive for multiple functions of these cNMPs. However, so far, the number of studies addressing the functions of cCMP and cUMP is very limited, apoptosis, vasodilation being emerging. Clearly, a systematic and unbiased approach is required to elucidate the function, and the experimental tools are in place. Considering the restricted occurrence of cCMP and cUMP in the kingdom of life, negative data on cCMP and cUMP functions in several systems are predicted. The existence of cCMP- and cUMP-producing toxins points to a role of these cNMPs in certain bacterial infections. Although sGC and sAC have been identified as NCs that are capable of producing cCMP and cUMP, it is doubtful that these are the only enzymes that produce noncanonical cNMPs. A broad approach, including genomic and systems biology methods will be required to identify novel NCs. Lastly, the cell type-specific cNMP patterns suggest that cNMPs operate in cooperation and not in isolation. Thus, the combination of cNMPs in functional experiments (be it in the natural cNMP form or the membrane-permeable cNMP-AM form) may provide critical information about the specific role of a given cNMP.

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cCMP and cUMP in Apoptosis: Concepts and Methods

Sabine Wolter, Fanni Dittmar, and Roland Seifert

Contents

Abstract

The cyclic nucleotides cAMP and cGMP are well-characterized second messenger molecules regulating many important intracellular processes, such as differentiation, proliferation, and apoptosis. The latter is a highly regulated process of programmed cell death wherein several regulatory proteins, like those belonging

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to the Bcl-2 family, are involved. The initiation of apoptosis is regulated by three different pathways: the intrinsic or mitochondrial, the extrinsic, and the ER stress pathway. Recently, it has been published that the pyrimidine cyclic nucleotides cCMP and cUMP also function as second messenger molecules, and additionally have an effect on apoptosis signaling pathways. cCMP induced PKA-independent apoptosis via the intrinsic and ER-stress pathway in S49 mouse lymphoma cells, and cCMP as well as cUMP induced apoptosis in human HEL cells via the intrinsic pathway. However, in human K-562 cells, which are known to be multidrug-resistant, cCMP and cUMP had no effect. Summarized in this chapter are the initiation of apoptosis by cCMP and cUMP regarding the various apoptotic pathways, the enzymes involved in apoptosis, as well as the most relevant methods for the detection and examination of apoptosis and the corresponding signaling pathways.

Keywords
Apoptosis • cCMP • cUMP • HEL cells • K-562 cells • PKA signaling • S49 cells

1 Introduction

Apoptosis is a cellular mechanism of programmed cell death that is characterized by membrane blebbing, condensation of the cytoplasm and nucleus, DNA fragmentation, and cell shrinkage. DNA fragmentation results in various DNA strand breaks and can be analyzed with the TUNEL (TdT-mediated dUTP-biotin nick end labeling) assay. This assay was formerly considered as the gold standard for the identification of apoptotic cells (Darzynkiewicz et al. 2008). Understanding the cellular apoptosis mechanisms can provide the opportunity to treat diseases or to develop drugs by targeting apoptotic genes and pathways.

The central enzymes involved in apoptosis are caspases (Hengartner 2000). Generally, three different pathways of apoptosis induction can be distinguished (Fig. 1):

(1) The extrinsic pathway is activated by death ligands like TNFα or FasL (Fas ligand) which bind to specific receptors. Afterwards, a so-called death-inducing signaling complex (DISC) is formed, the initiator caspase 8 is activated and triggers apoptosis by cleaving other downstream executioner caspases like caspase 3 and caspase 7, which can be analyzed by western blotting.
(2) The intrinsic mitochondrial pathway is initiated within the cell by irreparable genetic damage, severe oxidative stress, hypoxia, or deprivation of survival factors increasing the mitochondrial permeability caused by the loss of mitochondrial membrane potential. This leads to the release of pro-apoptotic factors, such as cytochrome c, from the mitochondrium into the cytoplasm. The release of these pro-apoptotic factors can be analyzed by flow cytometry after intracellular staining with an appropriate antibody, e.g., cytochrome c-FITC antibody. Cytochrome c triggers the formation of a complex known

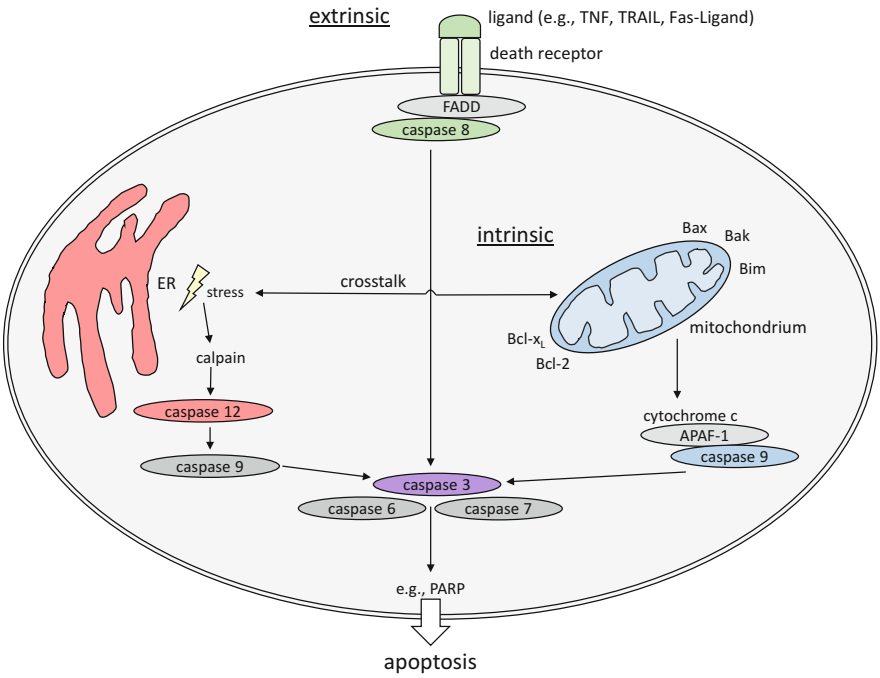

Fig. 1 Apoptotic pathways: extrinsic, intrinsic and ER stress-activated pathways

as apoptosome which is composed of cytochrome c, APAF-1 (apoptotic protease activating factor 1), and caspase 9. The apoptosome is able to activate caspase 3. Several proteins of the Bcl-2 family are important for the regulation of this pathway. These proteins have a pro- or anti-apoptotic function by promoting or blocking the mitochondrial release of pro-apoptotic factors (Martinou and Youle 2011). RNA or protein expression of the Bcl-2 family members can be analyzed by real-time PCR or western blotting, respectively.

(3) The third pathway is the intrinsic endoplasmatic reticulum (ER) pathway which leads to activation of caspase 12. This pathway is triggered by ER stress, e.g., by misfolded proteins (Szegezdi et al. 2003). Stimulation with brefeldin A (BFA), an inhibitor of intracellular protein transport, also induces ER stress. Thus, incubation of cells with BFA leads to a blockade of protein transport from the ER to the Golgi complex and an accumulation of proteins in the ER (Kaufman 1999). A potential cross-talk between the ER-induced apoptosis and the intrinsic pathway, controlled by Bcl-2 proteins, is under discussion (Häcki et al. 2000), and a function of several Bcl-2 proteins in ER stress has been reported as well (Siddiqui et al. 2015).

Besides apoptosis, other cell death mechanisms exist, namely autophagy and necrosis. Autophagy is a catabolic degradative process and is often associated with anti-proliferative mechanisms and physiological processes like differentiation, development, and cancer (Reggiori and Klionsky 2002). During autophagy, cytoplasmatic components are delivered to lysosomes and eliminated. Important enzymes involved in this process are autophagy-related genes (ATG) and LC3 (light chain 3) A/B proteins which therefore function as autophagy marker proteins. On the contrary, cells undergoing programmed necrosis show an increase in cell volume, swelling of organelles, and disruption of the cell membrane. Intracellular components are released and an inflammatory response is thereby often induced (Ouyang et al. 2012).

2 Cyclic Nucleotides and Induction of Apoptotic Pathways via Membrane-Permeant cNMP-Analogs

cNMPs with a free amino group at the nucleobase like cAMP, cGMP, and cCMP can be dibutyrylated (db-cNMPs). Such analogs are membrane-permeable. Other cNMPs like cUMP and also cIMP lack a free amino group and dibutyrylated analogs are therefore not available. Db-cNMPs are intracellularly cleaved by unspecific esterases or amidases into two metabolites and butyrate (Schwede et al. 2000). The most prevalent metabolite is the monobutyrylated form of the cNMP with a remaining butyryl residue at the nucleobase, the other metabolite with the butyryl residue at the ribose is formed to a much lower extent. Due to these issues, another group of membrane-permeant analogs has been developed: cNMP analogs with an acetoxymethyl ester residue at the ribose (cNMP-AMs). Similar to db-cNMPs, the acetoxymethyl ester analogs penetrate the membrane, and their ester residue is intracellularly cleaved by unspecific esterases. But in contrast to the cleavage products of the db-cNMPs, the only cleavage product of the cNMP-AMs is the unmodified cNMP (Beckert et al. 2014; Schultz 2003).

3 Function of PKA, cAMP, cGMP, and cUMP in Apoptosis

3.1 PKA in Apoptosis

The best known and extensively investigated cAMP target in eukaryotic cells is cAMP-dependent protein kinase (PKA) (Francis and Corbin 1999; Taylor et al. 2008). PKA is a tetrameric holoenzyme consisting of two identical regulatory subunits, each containing two binding sites for cAMP and two catalytic domains (Taylor et al. 2004). Without binding of cAMP, the enzyme is catalytically inactive. After activation, the catalytic subunits phosphorylate serine and threonine residues of specific target proteins, e.g., the transcription factor cyclic cAMP response element-binding protein (CREB) or the cAMP-responsive element modulator (CREM), thereby altering gene expression (Mayr and Montminy 2001).

3.2 cAMP in Apoptosis

cAMP functions both as pro- and anti-apoptotic factor, depending on the condition of the cells (Insel et al. 2012). In S49 lymphoma cells, cAMP acts as pro-apoptotic stimulus and operates via PKA to induce a cell cycle arrest in the G_1 phase and apoptosis (Yan et al. 2000). S49 cells are of thymic origin; the cells were derived from lymphoma of oil-treated BALB/c mice and established by van Daalen Wetters and Coffino (1987). In S49 kin$^-$ cells, mRNA for the catalytic subunits of PKA is expressed at a normal level, but the catalytic subunit protein is degraded rapidly. Therefore, the catalytic subunit is not detectable in S49 kin$^-$ cells at the protein level and, subsequently, the cells have no PKA activity (Orellana and McKnight 1990). As a consequence, S49 kin$^-$ cells are resistant to cAMP-induced apoptosis. Therefore, S49 cells are a useful tool to study the role of cAMP in apoptosis (Yan et al. 2000). Under basal conditions, cAMP and cCMP occur but neither cGMP nor cUMP are detectable in S49 cells (Hartwig et al. 2014).

Incubation of S49 wild-type (wt) cells with db-cAMP or other cAMP-increasing compounds induces a cell cycle arrest in the G_1 phase, followed by delayed cytolysis which finally leads to cell death (Yan et al. 2000). But after incubation with cAMP analogs or cAMP-increasing stimuli, S49 kin$^-$ cells show neither G_1 phase cell cycle arrest, nor mitochondria-dependent apoptosis, nor induction of PDE or other apoptosis-relevant proteins (Yan et al. 2000).

Surprisingly, apoptosis in S49 wt cells after treatment with cAMP-AM could not be detected (Wolter et al. 2015). This might be due to fast degradation of cAMP by phosphodiesterases (PDEs), since co-incubation with inhibitors of phosphodiesterases, like IBMX or rolipram, increased apoptosis in these cells. Moreover, activation of the PKA pathway and induction of apoptosis after treatment with PDE-resistant and cell-permeable cAMPS-AM analogs (*Sp*-cAMPS-AM and *Sp*-8-Br-cAMPS-AM) occurred in S49 wt cells as well (Wolter et al. 2015).

3.3 cGMP in Apoptosis

The best examined target of cGMP is cGMP-dependent protein kinase (PKG) (Hofmann 2005). cGMP acts as a pro-apoptotic factor in human breast cancer cell lines and in human cancer cells in general (Fallahian et al. 2011). However, stimulation with cGMP and cGMP-AM did not induce apoptosis in S49 wt or S49 kin$^-$ cells (Wolter et al. 2015). This could be due to rapid degradation of cGMP by PDEs or effective export via multidrug resistance proteins (MRPs).

Rat neuroblastoma B103 cells were used for a combination treatment for 48 h with high-concentrated cGMP-AM and cUMP-AM (both 200 μM). Prior to this treatment, cells were cultivated for 24 h in resting medium (RM), which had previously been used for studies on cCMP and cUMP generators (Hasan et al. 2014). After treatment, the cells became necrotic, whereas the individual substances had no effect and apoptosis could not be detected in B103 cells. However, based on the detection of

necrotic cells in the described study, it could be possible that cUMP as well as cGMP induce apoptosis in other cell lines or primary cells.

4 Hitherto Known Targets of cCMP and cUMP

cCMP is a cyclic pyrimidine nucleotide which binds to and activates cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) (Desch et al. 2010; Hammerschmidt et al. 2012; Wolter et al. 2011). cCMP regulates the function of HCN2 and HCN4 channels (DeBerg et al. 2016; Zong et al. 2012). So far, only phosphodiesterase (PDE) 7A1 was identified as cCMP-degrading enzyme (Monzel et al. 2014). Another mechanism for cCMP decrease in cells is the export via MRP 5 (Laue et al. 2014).

cUMP also activates PKA and PKG (Wolter et al. 2011) and regulates HCN2 and HCN4 channels. cUMP is degraded by PDE3A, 3B, and 9A (Reinecke et al. 2011), and is exported via MRP4 and MRP5 (Laue et al. 2014). Thus, cCMP and cUMP fulfill prerequisites for a second messenger (Seifert 2015).

The MRP inhibitor probenecid (Feller et al. 1995) can be used to investigate the role of MRPs in the apoptosis resistance of cancer cells. In HEL cells, which were established from a patient with erythroleukemia (Martin and Papayannopoulou 1982), the apoptosis-inducing effect of cUMP-AM could be enhanced after pretreatment with probenecid (Fig. 2). In K-562 cells, which were obtained from a

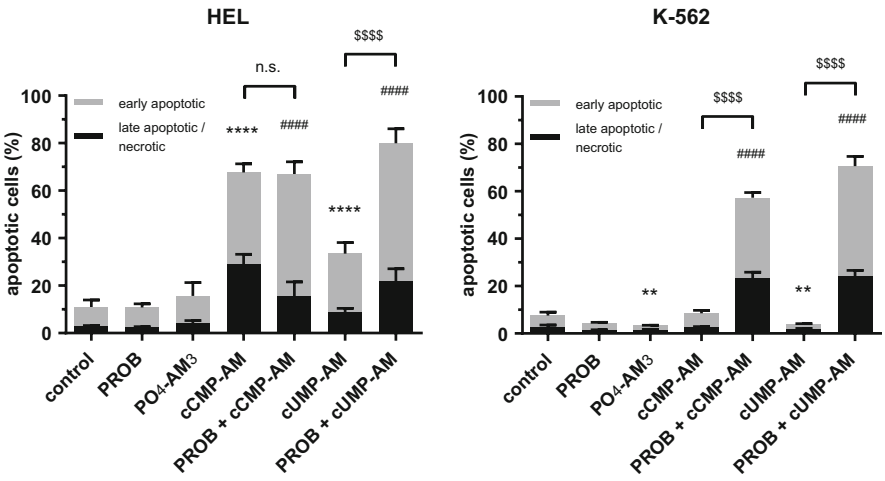

Fig. 2 Apoptosis induction after pretreatment with probenecid (PROB) in HEL and K-562 cells. 1×10^5 cells were treated with 150 µM cCMP-AM, 50 µM PO_4-AM_3, and 5 µM BFA for 24 h and 48 h, respectively. Apoptosis was measured by flow cytometry with a MACSQuant Analyzer. Shown are data as means ± SD. Data were analyzed by one-way ANOVA followed by Dunnett's test (****/####/$$$$, $p < 0.0001$; n.s., not significant, $p > 0.05$; $n = 4$). Data were adapted from Dittmar et al. (2016)

patient with chronic myelogenous leukemia (Lozzio and Lozzio 1975), pretreatment with probenecid resulted in apoptosis induction by cCMP-AM and cUMP-AM, whereas apoptosis was not induced without probenecid (Fig. 2) (Dittmar et al. 2016).

5 cCMP in Cell Proliferation and Apoptosis

First hints for a possible role of cCMP in the mechanism of cell proliferation arose from early experiments with mouse lymphocytic leukemia cells derived from ascetic fluid (L-1210 cells). In these experiments, performed by Bloch in 1974, cCMP enhanced cell proliferation in a dose-specific manner. A significant increase in cell proliferation was already obtained at 10 μM cCMP (Bloch 1974; Bloch et al. 1974). To determine the cell number, Bloch used the trypan blue exclusion method, which is a relatively simple method. Another method to determine cell proliferation is the incorporation of ^{3}H- or ^{14}C-thymidine (Adams 1980) or BrdU (Rothaeusler and Baumgarth 2007).

In contrast to the observations by Bloch et al. (Bloch 1974; Bloch et al. 1974), cCMP had no pro-proliferative effect, but inhibited cell proliferation and induced apoptosis in S49 wt and S49 kin^{-} cells (Wolter et al. 2015). Using S49 cells as a model system, hitherto known target proteins of cCMP like PKA, PKG, Epac, and HCN channels can be excluded in the apoptosis-inducing mechanism. These proteins could be excluded by real-time PCR, since mRNA of PKG, HCN2, and HCN4 channels was not expressed in S49 wt and S49 kin^{-} cells. Furthermore, stimulation of S49 cells with Epac-activating compounds did not alter cell proliferation (Wolter et al. 2015).

In S49 cells, cCMP induced apoptosis via the intrinsic apoptotic pathway and also via the ER stress pathway, whereas the extrinsic pathway was not involved. Induction of apoptosis was specific for cCMP-AM, because cAMP-AM, cGMP-AM, and cUMP-AM failed to activate apoptotic pathways in both S49 cell lines. Moreover, unmodified cCMP or other tested unmodified cNMPs (cAMP, cGMP, and cUMP) failed to induce apoptosis in S49 cells as well. These results indicate that extracellular effects like binding of cCMP to specific membrane receptors are not involved in the apoptosis-inducing mechanism of cCMP in S49 cells (Wolter et al. 2015).

In HEL cells, cCMP induced apoptosis also via the intrinsic apoptotic pathway. In contrast to S49 cells, induction of apoptosis was not specific for cCMP-AM, since cAMP-AM, cGMP-AM, and cUMP-AM activated the apoptotic pathway as well. Similar to S49 cells, unmodified cNMPs failed to induce apoptosis in HEL cells (Dittmar et al. 2016).

Recently, activation of the p44/p42 MAPK pathway by db-cCMP in mouse tissue lysates has been reported (Wolfertstetter et al. 2015). In contrast, activation of this pathway by cCMP-AM or db-cCMP in S49 cells was not detected. These differences could point to a cell type-specific activation pattern for cCMP, comparable to cAMP (Insel et al. 2012).

cCMP-induced apoptosis in S49 and HEL cells is caspase-dependent because the apoptotic mechanism was partly inhibited by the pan-caspase inhibitor Z-VAD-

FMK (Dittmar et al. 2016; Wolter et al. 2015). Since caspases are the relevant enzymes in apoptosis, the various mechanisms can be distinguished by their characteristic activated and cleaved caspases (Hengartner 2000). Caspases activated by cCMP were identified by western blot analysis for S49 cells (Fig. 3) (Wolter et al. 2015) and by a fluorometric caspase 3 activation assay for HEL cells (Fig. 4).

Furthermore, intracellular cCMP also activated caspases 9 and 12 in S49 cells (Wolter et al. 2015). Caspase 12 is involved in the ER stress apoptotic pathway (Nakagawa et al. 2000). The extrinsic apoptotic pathway is not involved in cCMP-promoted apoptosis, since activation of caspase 8 was not detected after cCMP-AM treatment. BFA activates the ER stress apoptotic pathway and was therefore used as a negative control in the caspase 8 activation assay (Kaufman 1999), whereas a combination of cycloheximide and tumor necrosis factor (TNF) α was used as positive control (Fig. 5).

The involvement of the mitochondria-dependent apoptosis pathway was investigated by the depolarization of the mitochondrial membrane potential (Δψ) in HEL cells after treatment with cCMP-AM (Fig. 6) (Dittmar et al. 2016).

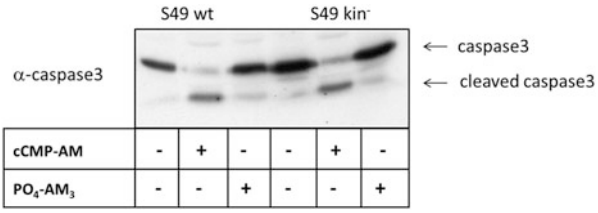

Fig. 3 Activation of the executioner caspase 3 after cCMP-AM treatment in S49 wt and S49 kin$^-$ cells. 1×10^6 cells were treated with 150 μM cCMP-AM and 50 μM PO_4-AM_3 for 20 h. Expressions of caspase 3 and cleaved caspase 3 were analyzed using western blotting. Data were adapted from Wolter et al. (2015)

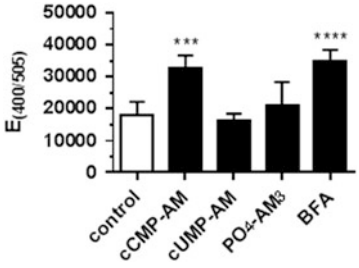

Fig. 4 Activation of the executioner caspase 3 after cCMP-AM and cUMP-AM treatment in HEL cells. 8×10^5 cells were treated with 150 μM cCMP-AM, 50 μM PO_4-AM_3, and 5 μM BFA for 20 h. Caspase 3 activity was measured at 400 nm excitation and 505 nm emission in a fluorometer. Shown are data as means ± SD. Data were analyzed by one-way ANOVA followed by Dunnett's test (***$p < 0.001$; ****$p < 0.0001$; $n = 5$). Data were adapted from Dittmar et al. (2016)

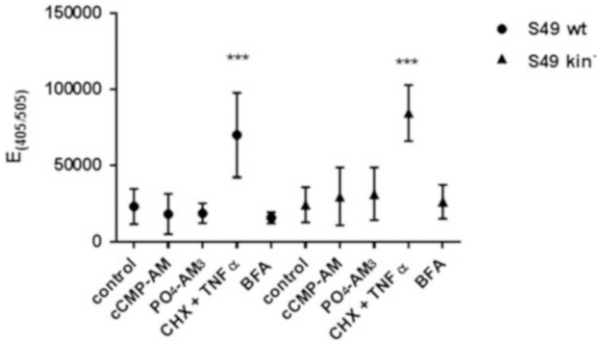

Fig. 5 No activation of caspase 8 after cCMP-AM treatment in S49 wt and S49 kin$^-$ cells. 1×10^6 cells were treated with 150 μM cCMP-AM, 50 μM PO_4-AM_3, and 4 μM BFA for 20 h. As a positive control, cells were also incubated with a combination of 100 ng/ml cyclohexemide and 1 ng/ml TNFα μM for 4 h. Caspase 8 activity was measured at 400 nm excitation and 505 nm emission in a fluorometer. Shown are data as means ± SD. Data were analyzed by one-way ANOVA followed by Dunnett's test (***$p < 0.001$; $n = 5$). Data were adapted from Wolter et al. (2015)

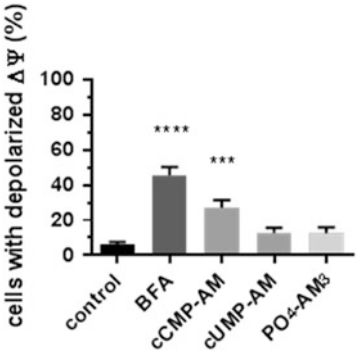

Fig. 6 Loss of mitochondrial membrane potential after cCMP-AM treatment in HEL cells. 6×10^5 cells were treated with 150 μM cCMP-AM, 50 μM PO_4-AM_3, and 5 μM BFA for 24 h. Mitochondrial membrane potential was analyzed by flow cytometry. Shown are data as means ± SD. Data were analyzed by one-way ANOVA followed by Dunnett's test (***$p < 0.001$; ****$p < 0.0001$; $n = 3$). Data were adapted from Dittmar et al. (2016)

Caspase 9 is an initiator caspase of the intrinsic apoptotic pathway (Elmore 2007). The importance of this pathway for cAMP-induced apoptosis in S49 wt cells and the release of cytochrome c were shown by Zhang et al. (2008). cCMP also induced cytochrome c release in these cells, as shown by flow cytometry analysis with a specific antibody (Fig. 7). Surprisingly, cytochrome c release was also detected in S49 kin$^-$ cells, but to a lesser extent (Fig. 7). PKA activity is not absolutely required for cytochrome c release, but could be responsible for the stronger release of cytochrome c by cCMP in S49 wt cells, since S49 kin$^-$ cells lack PKA (Orellana and McKnight 1990).

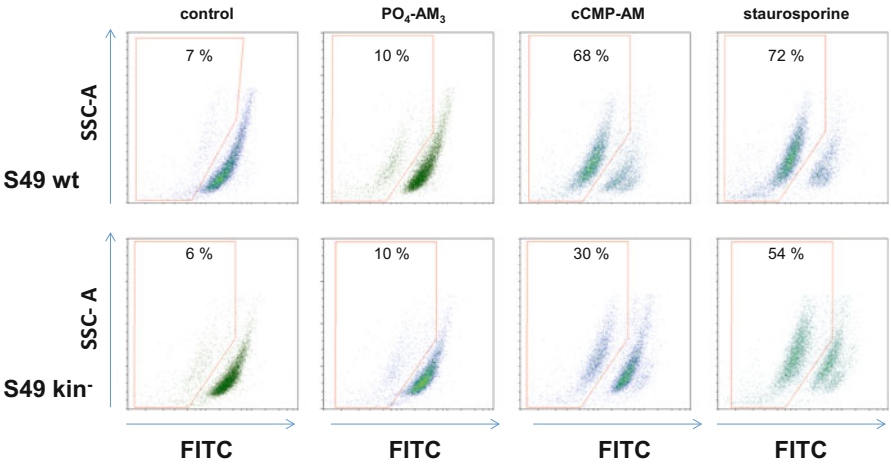

Fig. 7 Cytochrome c release after cCMP-AM treatment. 5×10^5 cells were treated with 50 µM PO_4-AM_3, 150 µM cCMP-AM and 0.05 µM staurosporine for 24 h. After fixation, the S49 wt and S49 kin^- cells were stained with an anti-cytochrome c-FITC antibody and analyzed by flow cytometry. Shown are the SSC-A (side scatter-area) and the FITC signal of representative dot blots. Data were adapted from Wolter et al. (2015)

Table 1 cCMP-AM induces apoptosis in several cell lines: analyzed by flow cytometry

Cell line	Species	Tissue	Description
S49[a]	Mouse	Thymocyte	T-lymphoma
Jurkat	Human	T-lymphocyte	Acute T-cell leukemia
MOLT-3	Human	T-cell	Acute lymphoblastic leukemia (ALL)
U937	Human	Lung	Histiocytic lymphoma
Hut	Human	T-lymphocyte	T-cell lymphoma
PLB-985	Human	Promyeloblast	Acute myeloid leukemia (AML)
HEL 92.1.7[b]	Human	Blood	Erythroleukemia

1×10^5 cells/ml were treated with 100 µM cCMP-AM and 33 µM PO_4-AM_3 as control in a volume of 2 ml for 48 h. The cells were sedimented by centrifugation, stained with annexin V-APC/PI, and analyzed by flow cytometry with a MAQS Quant Analyzer (Miltenyi Biotech, Bergisch Gladbach, Germany)

[a]Published in Wolter et al. (2015)

[b]Dittmar et al. (2016). All other results are unpublished data

Additional cell lines from human, mouse, rat, and insect species were treated with cCMP-AM and analyzed by flow cytometry. PO_4-AM_3 was used as a control for the acetoxymethyl residue. Thus, cell lines that are resistant to cCMP-AM treatment and cell lines that become apoptotic due to treatment with cCMP-AM were identified (summarized in Tables 1 and 2).

Comparative analysis of these results could help to identify hitherto unknown cCMP target protein molecules. In addition, highly active PDEs and/or MRPs could account for the apoptosis resistance of certain cell lines.

Table 2 Cell lines resistant to cCMP-AM-induced apoptosis: analyzed by flow cytometry

Cell line	Species	Tissue	Description
K-562[a]	Human	Blood	Chronic myelogenous leukemia (blasts)
HEK293	Human	Kidney	Embryonic, epithelial
B103	Rat	Brain	Neuroblastoma
Sf9	Insect (*Spodoptera frugiperda*)	Ovarian	
EL4	Mouse	T-lymphocyte	Ascites lymphoma lymphoblast
LBRM	Mouse	T-lymphocyte	Lymphoma lymphoblast

1×10^5 cells/ml were treated with 100 μM cCMP-AM and 33 μM PO_4-AM_3 as control in a volume of 2 ml for 48 h. The cells were sedimented by centrifugation, stained with annexin V-APC/PI, and analyzed by flow cytometry with a MAQS Quant Analyzer (Miltenyi Biotech, Bergisch Gladbach, Germany)

[a]Modified conditions, published in Dittmar et al. (2016). All other results are unpublished data

5.1 Relevant Genes in Apoptosis

During apoptosis, expression of a broad range of genes belonging to the Bcl-2 protein family, proteins related to p53, or cyclin-dependent kinases is altered (Dlamini et al. 2015). Their expression can be analyzed by real-time PCR. After RNA preparation, cDNA is reversely transcribed using moloney murine leukemia virus reverse transcriptase (MLV-RT) or another appropriate enzyme. Sybr Green and gene-specific primers for relevant genes in apoptosis and a housekeeping gene (e.g., Actb, Gus, or HPRT) or TaqMan probes can be used to analyze mRNA expression. Analysis of real-time PCR data and determination of gene expression alteration can be evaluated using the $\Delta\Delta C_t$ equitation (Livak and Schmittgen 2001).

After different exposure times of S49 wt and S49 kin^- cells with cCMP-AM and PO_4-AM_3, expression of Bim and other important apoptotic genes was analyzed by quantitative real-time PCR. The pro-apoptotic protein Bim belongs to the Bcl-2 protein family and was induced after cAMP stimulation in S49 wt cells (Zhang and Insel 2004). No significant induction of Bim or Bcl-2 was detectable in S49 wt or in S49 kin^- cells after treatment with cCMP-AM, but other apoptotic relevant genes were induced in S49 wt cells, since mRNA expression of Gadd45α, cFos, and Nr4A1 was increased. On the contrary, no increase in mRNA expression of analyzed genes was detected in S49 kin^- cells. Treatment with the control substance PO_4-AM_3 did not alter gene expression in both cell lines (Wolter et al. 2015).

5.2 Cell Cycle Analysis: G_1 Arrest

Yan et al. showed that isoproterenol and also cAMP caused cell cycle arrest at G_1 and increased the number of dead S49 wt cells (Yan et al. 2000). Cell viability was reduced after 36 h of treatment and the majority of S49 wt cells were dead after

72 h. cCMP-AM induced apoptosis in S49 wt and S49 kin$^-$ cells much faster, but no G_1 arrest could be determined by cell cycle analysis. Forskolin (Fsk) was used as a positive control for cell cycle analysis and increased the subG$_1$ and $G_{0/1}$ population in S49 wt cells, but not in S49 kin$^-$ cells as expected (Fig. 8a, b, unpublished data).

Cell cycle analysis was also performed with higher concentrations (up to 200 µM cCMP-AM) and for longer time periods of treatment. The results were similar: G_1

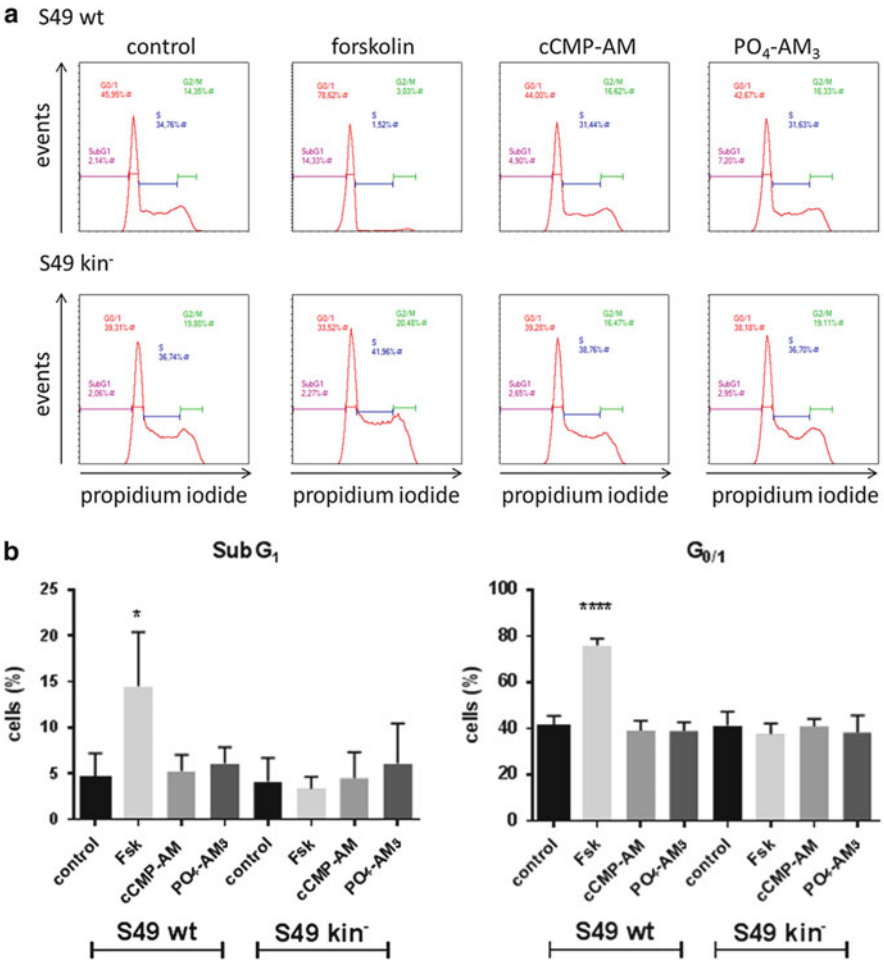

Fig. 8 (**a**) Representative histograms of original flow cytometric data. S49 wt and S49 kin$^-$ cells were incubated with 30 µM forskolin (Fsk), 100 µM cCMP-AM, and 33 µM PO_4-AM_3 for 24 h. Cells were fixed with ethanol, digested with RNase A, and stained with PI. Cell cycle analysis was performed using flow cytometry where results are displayed as a histogram. (**b**) Cell cycle analysis of S49 wt and S49 kin$^-$ cells. Intervals for the different phases in the histograms were used to calculate statistics for subG$_1$ and $G_{0/1}$. Shown are data as means ± SD. Data were analyzed by one-way ANOVA followed by Bonferroni's post-test (*$p < 0.05$; ****$p < 0.0001$; $n = 3$)

arrest was evident neither in S49 wt nor in S49 kin$^-$ cells. These differences after treatment with forskolin and cCMP-AM for cell cycle analysis are additional indications for a specific mechanism. Hitherto known cCMP targets were already excluded as targets for induction of apoptosis by cCMP, indicating the existence of other targets and mechanisms which should be investigated using S49 kin$^-$ cells as model system.

However, cell cycle analysis of HEL cells after treatment with cCMP-AM and cUMP-AM showed different results: SubG$_1$ population was increased by cCMP-AM while G$_2$/M population was simultaneously decreased (Fig. 9), whereas G$_{0/1}$ and S populations were not affected, suggesting a cell cycle block at the G$_2$/M checkpoint (Dittmar et al. 2016).

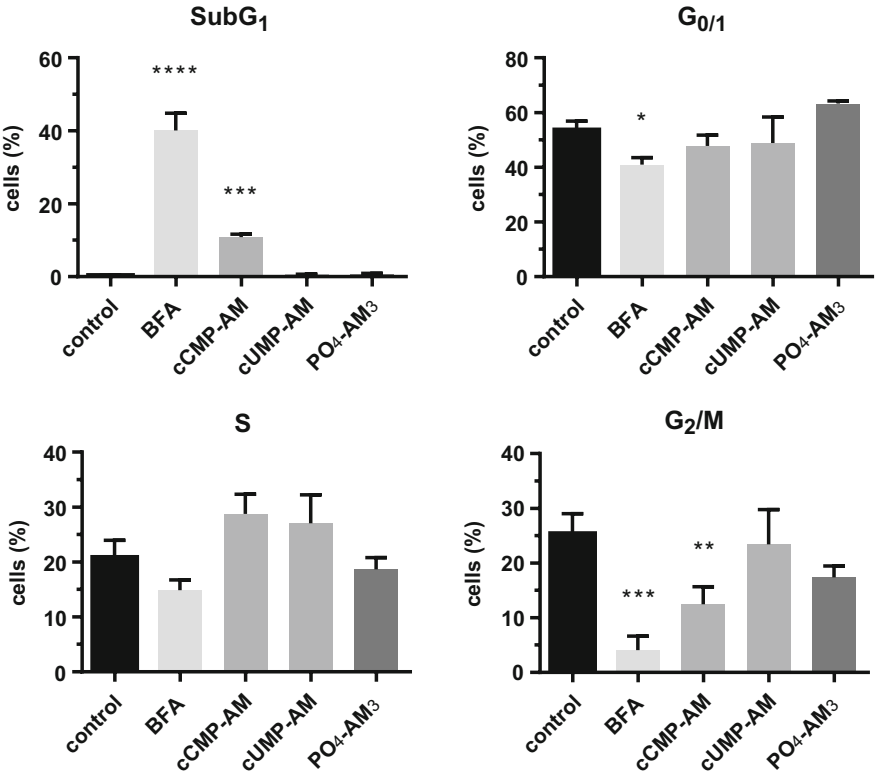

Fig. 9 Cell cycle analysis of HEL cells. 5×10^5 cells were treated with 150 µM cCMP-AM, 50 µM PO_4-AM_3, and 5 µM BFA for 24 h. Samples were analyzed by flow cytometry with a MACSQuant Analyzer. Shown are data as means ± SD. Data were analyzed by one-way ANOVA followed by Dunnett's test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; $n = 3$). Data were adapted from Dittmar et al. (2016)

5.3 Interaction of cCMP and cUMP with Bcl-2 Family Proteins and Induction of Apoptosis

Proteins belonging to the Bcl2-family play a very important role in apoptosis. Direct binding between cCMP or cUMP and Bcl-2 proteins can potentially occur, or it is possible that cCMP and cUMP induce or repress mRNA expression of these proteins. So far, no proteins belonging to the Bcl-2 family could be identified using agarose-matrices as cCMP- or cUMP-binding proteins (Hammerschmidt et al. 2012). However, for cAMP-mediated apoptosis in S49 wt cells, mRNA expression of the pro-apoptotic protein Bim was reported (Zhang and Insel 2004). Increase of Bim mRNA expression is PKA-dependent, because it was not detectable in S49 kin^- cells. cCMP treatment did not alter mRNA expression of Bim or Bcl-2 (Wolter et al. 2015), supporting the concept of PKA-independent mechanisms of action for cCMP. More proteins belonging to the Bcl-2 family with pro- or anti-apoptotic function need to be examined after cCMP- and cUMP-treatment and additional cell lines should be used as well.

6 Experimental Methods

6.1 Determination of Cell Number and Cell Proliferation

6.1.1 Cell Number: Cell Counting After Trypan Blue Staining

Trypan blue is one of the several stains recommended for use in dye exclusion procedures for viable cell counting and was first mentioned by Weisenthal et al. (1983). This method is based on the principle that viable cells do not take up impermeable dyes like trypan blue, whereas dead cells are permeable and take up the dye. Therefore, viable cells can be distinguished from dead cells using this method. The cell number is estimated using a hemacytometer. This method for determination of the cell number has been used for cCMP-AM-treated S49 (Wolter et al. 2015), HEL and K-562 cells (Dittmar et al. 2016).

6.1.2 Cell Proliferation: BrdU Incorporation

Four different assays are used to evaluate cell proliferation: (1) assays measuring incorporation of radioactive thymidine (^{3}H thymidine) or its indirectly fluorescent-labeled analog (5-bromodeoxyuridine, BrdU) into newly synthesized DNA of proliferating cells, (2) assays utilizing cells labeled with a fluorescent dye (carboxyfluorescin diacetate succinimidyl ester, CFSE) that measure the loss of the fluorescent-labeled proteins during cell division, (3) assays measuring unique markers expressed in dividing cells (e.g., Ki-67) only, and (4) assays measuring biochemical activity of cells as a substitute for proliferation (MTT) (Rothaeusler and Baumgarth 2007). The advantage of BrdU labeling compared to the others is that it constitutes a non-radioactive labeling technique which avoids costly and time-consuming cell isolation procedures (Rothaeusler and Baumgarth 2007). The halogenated pyrimidine analog BrdU, which substitutes thymidine during DNA synthesis and becomes

stably integrated, can thus be detected for many months either (a) cytochemically, (b) immunocytochemically, (c) by selective photolysis, or (d) by flow cytometry (Dolbeare et al. 1983).

(a) Cytochemical methods of BrdU detection were previously described by Latt in 1973 (Latt 1973) and by Darzynkiewicz et al. in 1978 (Darzynkiewicz et al. 1978).

(b) Immunodetection of BrdU using specific monoclonal antibodies (mAbs), developed by Gratzner in 1982 (Gratzner 1982), allows labeling of cells in the S phase of the cell cycle (Darzynkiewicz and Juan 2001). The most common technique of cell cycle analysis based on BrdU incorporation into double-stranded DNA is the immunocytochemical detection of BrdU combined with simultaneous measurement of cellular DNA content followed by bivariate data analysis (Dolbeare et al. 1983). Since incorporated BrdU in nuclear chromatin is inaccessible to the anti-BrdU antibody, certain criteria have to be considered: (1) cellular and nuclear membranes must be fixed and permeabilized to allow access of the anti-BrdU antibody to the BrdU-labeled DNA; (2) the double-stranded DNA has to be digested to generate single-stranded DNA that is accessible to binding by the detection antibody; (3) the method has to be gentle enough to avoid cell aggregation and distortion of cell morphology (Rothaeusler and Baumgarth 2007).

One major drawback of BrdU detection procedures is the necessity for fixing and permeabilizing cells (Rothaeusler and Baumgarth 2007). The partial DNA denaturation by exposure of cells to heat or strong acid often results in cell damage, aggregation (clumping), and significant cell loss (Darzynkiewicz and Juan 2001). Besides, while induction of DNA denaturation by acid may prove to be satisfactory with one cell type, it may fail with another, since some cell types require higher acid concentration (e.g., 4 M HCl vs 2 M HCl) for optimal results, which also applies for thermal DNA denaturation (Darzynkiewicz and Juan 2001). Cell adherence to tube surfaces is another critical factor (Darzynkiewicz and Juan 2001).

In 1992, Carayon and Bord showed that using bovine pancreatic DNase I instead of hydrochloric acid for DNA breakup and Tween-20/p-formaldehyde instead of ethanol for fixation, PE and APC fluorescent signals could be maintained and cells could be labeled with anti-BrdU antibodies (Carayon and Bord 1992). Nowadays, commercially available fixation/permeabilization solutions that fix and permeabilize in a single step are mainly used (Rothaeusler and Baumgarth 2007). Afterwards, a BrdU mAb is added to detect the incorporated BrdU and a horseradish peroxidase (HRP-) linked secondary antibody is used to recognize the bound detection antibody. Chemiluminescent reagent is then added for signal development. The magnitude of light emission, which is measured in relative light units (RLU), is proportional to the quantity of BrdU incorporated into cells and is therefore a direct indication of cell proliferation.

(c) Another approach for BrdU detection is based on selective photolysis of DNA containing the incorporated BrdU. Photolytically generated DNA strand breaks are subsequently labeled with fluoresceinated nucleotides. This reaction is catalyzed by exogenous terminal transferase and also known as DNA strand break induction by photolysis (SBIP) (Li et al. 1996). Since DNA denaturation is not required in SBIP, the procedure can be combined with either immunocytochemical analysis – e.g., for cell immunophenotyping together with analysis of DNA replication – or with simultaneous detection of apoptotic cells (Li et al. 1996).

(d) BrdU can be used to assess proliferating cell populations by flow cytometry or fluorescent microscopy. Therefore, DNA that has previously incorporated BrdU is detected by using a fluorochrome-conjugated anti-BrdU antibody (Leif et al. 2004). To avoid unspecific staining, it is necessary to use a dye as live/dead cell discriminator (e.g., propidium iodide, PI) (Rothaeusler and Baumgarth 2007). BrdU-immunostained nuclei can be counted to generate a labeling index analogous to thymidine autoradiography (Boulton and Hodgson 1995).

BrdU has been widely applied in vitro for cultured cells and tissues (Boulton and Hodgson 1995), but in vivo applications must be carefully controlled since toxicity issues can arise with long-term BrdU application (Reome et al. 2000). Thus, its use in human tissue is limited to ex vivo studies (Boulton and Hodgson 1995). Since BrdU is a potential carcinogen and can be absorbed through skin or by inhalation (Darzynkiewicz and Juan 2001), special precautions are required for its laboratory handling (Boulton and Hodgson 1995). Furthermore, BrdU solutions are light-sensitive and should be protected from light during storage and handling (Darzynkiewicz and Juan 2001).

6.1.3 Thymidine Incorporation

The thymidine incorporation assay uses a strategy wherein a radioactive nucleoside, ^{3}H-thymidine or ^{14}C-thymidine, is incorporated into the replication strands of chromosomal DNA during mitotic cell division. For measurement of the radioactivity in DNA recovered from the cells in order to define the extent of cell division, cell cultures are typically set up in microplates. The labeled DNA is usually captured with a cell harvester on glass fiber filter discs which are then placed in liquid scintillation counting vials or directly harvested into a filter plate for counting on a scintillation beta-counter. Further details are summarized by Adams (1980).

6.1.4 TUNEL Assay

The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay is based on fluorochrome labeling of 3′-OH termini of DNA strand breaks which represent a characteristic hallmark of apoptosis and occur at later stages of the apoptotic process. This assay was first described in 1992 by Gavrieli et al. (1992). Cells undergoing apoptosis showed cleavage of their genomic DNA to approximately 180 bp fragments as a result of endonuclease activation. These strand breaks

were afterwards labeled in situ, either in individually fixed, permeabilized cells or in tissue sections by in situ nick translation using the TUNEL technique. The reaction is relatively specific and detects apoptotic nuclei in tissues and at the single-cell level (Gavrieli et al. 1992). Due to difficulties in discriminating between apoptotic and necrotic cells, the assay has been improved: New methods use the incorporation of dUTPs, modified with fluorophores, haptens, biotin, or bromine, which can be detected directly, when a fluorophore is added, or indirectly with streptavidin or antibodies.

6.2 Detection of Apoptosis Using Flow Cytometry

6.2.1 Annexin V/Propidium Iodide Staining

Apoptotic cells change the structure by redistribution of phosphatidylserine from the internal to the external membrane surface, which can be used as an indicator for early apoptotic cells. Annexin V interacts specifically and strongly with the exposed phosphatidylserine in the presence of physiological calcium (Ca^{2+}) concentration (Moore et al. 1998), whereas propidium iodide (PI) binds to DNA by intercalating between the bases without sequence preference. Once PI is bound to DNA, its fluorescence is enhanced up to 30-fold. Since PI is membrane-impermeable, it intercalates only in the DNA of cells with lost membrane integrity. Therefore, simultaneous staining of cells with fluorophore-labeled annexin V (e.g., annexin V-APC) and PI allow the discrimination between early apoptotic and necrotic cells by flow cytometry (Moore et al. 1998). This method was used for cCMP-AM and cUMP-AM-treated S49 (Wolter et al. 2015), HEL and K-562 cells (Dittmar et al. 2016). For other cell lines, the results regarding this method are unpublished data (Tables 1 and 2).

6.2.2 Intracellular Staining with Cytochrome c-FITC-Antibody

During mitochondrial apoptosis, cytochrome c is redistributed from mitochondria to the cytosol in intact cells (Goldstein et al. 2000). The redistribution can be determined by flow cytometry using an antibody against cytochrome c combined with a fluorophore (e.g., fluorescein isothiocyanate, FITC). Therefore, cells are fixed with formaldehyde to stabilize the cell membrane and permeabilized with a detergent like saponin or alcohol to enable antibodies against intracellular proteins to reach their antigen. A significant increase in the lower FITC-signal is a criterion for cytochrome c release from the mitochondria and indicates the involvement of the intrinsic apoptotic pathway. The results regarding cCMP-AM treated S49 cells were published in Wolter et al. (2015).

6.2.3 Cell Cycle Analysis

Cell cycle analysis by quantitation of DNA content was one of the earliest applications of flow cytometry in apoptosis. The first protocol for cell cycle analysis using PI-staining was presented in 1975 by Awtar Krishan (Krishan 1975). Besides PI, other fluorophores, e.g., 4′,6-diamidine-2′-pheylindole (DAPI), which binds

also stoichiometrically to the DNA, can be used to determine the cell cycle phase of apoptotic cells (Darzynkiewicz et al. 1992). Depending on the cell cycle phase, the amount of DNA varies and correlates with the fluorescence intensity. Four distinct phases of the cell cycle can be distinguished: G_1, S, G_2, and M phase. During the S phase, DNA of the cells is duplicated. However, discrimination between G_2 and M phase cannot be performed based on their DNA content (Nunez 2001). By means of special mathematical models, the relative amount of cells in the different phases of the cell cycle can be represented graphically (Figs. 7a, b and 8). Often, cells have to be fixed using alcohol (e.g., ethanol) or to be permeabilized with a detergent to allow the fluorophore to enter the cells. Since the dye can also bind to RNA, the cells have to be treated with RNase after fixation. This method was used for HEL cells (Dittmar et al. 2016). Unpublished data for S49 cells are shown in Fig. 8.

6.3 Determination of the Mitochondrial Membrane Potential

Mitochondria play an important role in many pathophysiological conditions and are involved in the intrinsic mitochondria-dependent apoptosis pathway (Salvioli et al. 2000). Therefore, determining the status of this organelle can be used as a tool to examine apoptosis in cells by analyzing the mitochondrial membrane potential ($\Delta\psi$) which arises from the net movement of positive charge across the inner mitochondrial membrane (Mathur et al. 2000). The status of $\Delta\psi$ is evaluated by using the fluorescent cyanine dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimid-azolcarbocyanine iodide) (Reers et al. 1995; Smiley et al. 1991), where JC-1 stands for *1st J-aggregate-forming cationic dye found to be sensitive to* $\Delta\psi$. JC-1 has been generally considered as a reliable and sensitive fluorescent probe for detecting differences in $\Delta\psi$ due to its dual emission characteristics (Reers et al. 1991, 1995; Salvioli et al. 2000). It produces two fluorescence emission peaks congenial to the two physical forms of the dye. (1) The monomer, which is the predominant form at low concentrations of the dye or at low $\Delta\psi$, has a fluorescence maximum of 520 nm after excitation at 490 nm and emits green fluorescence. (2) The so-called "J-aggregate", which is the predominant form at high concentrations of dye or high $\Delta\psi$, has a fluorescence maximum of 585 nm after excitation at 490 nm and emits orange-red fluorescence (Mathur et al. 2000; Smiley et al. 1991). Both colors can be detected using the filters commonly mounted in almost all confocal microscopes and flow cytometers, so that green emission can be analyzed in one fluorescence channel and orange-red emission in the other (Cossarizza et al. 1993).

In viable cells whose $\Delta\psi$ is polarized, JC-1 is rapidly taken up by mitochondria, leading to an increase of JC-1 concentration and the formation of the so-called J-aggregates, whereas in mitochondria with depolarized $\Delta\psi$ (which is associated with apoptosis) JC-1 does not accumulate but remains in the cytoplasm as monomers. Therefore, apoptotic cells emit mostly green fluorescence, whereas viable cells emit relatively high levels of both green and orange-red fluorescence (Facompré et al. 2000; Mathur et al. 2000; Petit et al. 1995).

The poor water solubility of JC-1 makes it hard to use it for some applications. Therefore, JC-10 has been developed as an alternative when high dye concentration is desired. Compared to JC-1, JC-10 has a much better water solubility. In some cell lines, JC-10 has even superior performance to JC-1. This method was used for cCMP-AM- and cUMP-AM-treated HEL cells (Dittmar et al. 2016).

6.4 Detection of Caspases by Western Blotting and Fluorometric Assays

Caspases are the most important enzymes in apoptosis (Elmore 2007). They recognize a 4 or 5 amino sequence of the target substrate which includes an aspartic acid residue and cleave the substrate protein. This occurs at the carbonyl end of the aspartic acid residue. Activation of specific initiator caspases indicates the three different apoptotic pathways. Identification of caspases can be performed by immunoprecipitation or western blotting techniques. With specific antibodies against cleaved caspase products or against the full-length proteins, the different caspases can be identified. This requires very specific antibodies which can discriminate between the different caspases; sources of antibodies are documented in Dittmar et al. (2016) and Wolter et al. (2015). Western blotting was used for S49 and HEL cells treated with cCMP-AM and cUMP-AM (Dittmar et al. 2016; Wolter et al. 2015).

Additional methods for determination of the involvement of specific caspases are calorimetric or fluorometric assays. For calorimetric assays, a substrate with a specific recognition sequence (e.g., a tetrapeptide sequence DEVD) of the respective caspase linked to a chromophore (e.g., p-nitroaniline) is used (Gurtu et al. 1997). After cleavage, light emission of the chromophore increases and can be quantified using a spectrophotometer. For fluorometric assays, fluorochrome substrates are used which become fluorescent after cleavage of the substrate. For example, a caspase 8 fluorometric assay is based on the detection of AFC (7-amino-4-trifluoromethyl coumarin) after cleavage of the labeled substrate IETD-AFC. For detection of caspase 3 activation, the substrate DEVD-AFC can be used. Measurement of caspase 3 and caspase 8 activity was used for HEL and S49 cells, respectively (Dittmar et al. 2016; Wolter et al. 2015).

7 Conclusion and Further Perspectives

Recently, Wilderman et al. published new insights regarding cAMP-mediated apoptosis (Wilderman et al. 2015). They used quantitative proteomic analysis of mitochondria-enriched fractions and found some upregulated genes for enzymes involved in BCAA (branched-chain amino acid) and fatty acid oxidation in S49 wt cells after stimulation with a cAMP analog. cAMP acts via PKA and causes mitochondrial perturbation which leads to apoptosis in S49 wt cells (Wilderman et al. 2015).

Based on this new level of knowledge, further experiments concerning the cCMP mechanism could be performed, although the required high concentrations of cCMP-AM would be a limitation, when similarly adapted to the cAMP concentrations used by Wilderman et al. (2015). Therefore, it is necessary to develop additional cCMP analogs that can be used in lower concentrations than cCMP-AM. Interestingly, Wilderman et al. performed a proteomic approach, but could not find a single gene whose expression is altered in S49 kin$^-$ cells. Such an approach using S49 kin$^-$ cells and cCMP-AM should be performed, because in contrast to cAMP, cCMP induced apoptosis in both S49 cell lines and hitherto known cCMP targets are excluded in S49 kin$^-$ cells. The identification of unique cCMP target molecules would support the establishment of cCMP as an independent second messenger.

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Mammalian Nucleotidyl Cyclases and Their Nucleotide Binding Sites

Stefan Dove

Contents

Abstract

Mammalian membranous and soluble adenylyl cyclases (mAC, sAC) and soluble guanylyl cyclases (sGC) generate cAMP and cGMP from ATP and GTP, respectively, as substrates. mACs (nine human isoenzymes), sAC, and sGC differ in their overall structures owing to specific membrane-spanning and regulatory domains but consist of two similarly folded catalytic domains C1 and C2 with high structure-based homology between the cyclase species. Comparison of available crystal structures – VC1:IIC2 (a construct of domains C1a from dog mAC5 and C2a from rat mAC2), human sAC and sGC, mostly in complex with substrates, substrate analogs, inhibitors, metal ions, and/or modulators – reveals that especially the nucleotide binding sites are closely related. An evolutionarily well-conserved catalytic mechanism is based on common binding modes, interactions, and structural transformations, including the participation of two metal ions in catalysis. Nucleobase selectivity relies on only few mutations. Since in all cases the nucleoside moiety is embedded in a relatively spacious cavity, mACs, sAC, and sGC are rather promiscuous and

Molecular models presented in Figs. 3–9 were generated and drawn with the program suite SYBYL X2 (Certara LP, Princeton, NJ, USA).

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bind nearly all purine and pyrimidine nucleotides, including CTP and UTP, and many of their derivatives as inhibitors with often high affinity. By contrast, substrate specificity of mammalian adenylyl and guanylyl cyclases is high due to selective dynamic rearrangements during turnover.

Keywords
Binding modes · Inhibitors · Membranous adenylyl cyclase · Soluble adenylyl cyclase · Soluble guanylyl cyclase · Structure · Substrates

1 Introduction

Nucleotidyl cyclases generate canonical cyclic nucleotides (cNMPs) – cAMP, cGMP – from ATP and GTP as substrates. In the context of a book on noncanonical cNMPs, the possible role of pyrimidine nucleotides and their derivatives as substrates and inhibitors of nucleotidyl cyclases is of interest. Structural aspects related to mechanisms of binding and catalysis, substrate specificity, and ligand selectivity come into play. The question arises: why and to what extent are nucleotidyl cyclases promiscuous? Analysis of their nucleotide binding sites and of enzyme-ligand interactions may help to answer this question.

The main scope of this chapter is to describe and to discriminate nucleotide binding sites of mammalian nucleotidyl cyclases on the basis of known crystal structures rather than to deal with the whole field of membranous and soluble cyclase species. Structures of the catalytic domains of three mammalian nucleotidyl cyclases have been released up to now, namely:

- A construct of catalytic domains C1a from dog membranous adenylyl cyclase (mAC) type 5 (VC1) and C2a from rat mAC type 2 (IIC2) in complex with $G_{s\alpha}$ and ATP analogs (Mou et al. 2009; Tesmer et al. 1997, 1999), with P-site inhibitors and pyrophosphate (Dessauer et al. 1999; Tesmer et al. 2000), and with 2′,3′-substituted purine nucleotide inhibitors (Hübner et al. 2011; Mou et al. 2005, 2006)
- Catalytic domain of human soluble adenylyl cyclase type 10 (sAC) in complex with substrate analogs, cAMP, pyrophosphate, bicarbonate, and inhibitors (Kleinboelting et al. 2014a, 2014b; Saalau-Bethell et al. 2014)
- Heterodimeric catalytic domains $sGC\alpha_{cat}$:$sGC\beta_{cat}$ of human soluble guanylyl cyclase (sGC) (Allerston et al. 2013; Seeger et al. 2014)

Comparison of the three enzymes is intriguing: similar cores – two catalytic domains (C1, C2) responsible for cNMP formation – are components of quite different proteins. According to location (membrane associated or dissolved in the cytoplasm) and to intrinsic regulatory functions, each enzyme contains additional specific domains enabling, e.g., membrane spanning or binding of modulators. Moreover, mAC and sAC are single protein chains, whereas sGC is a heterodimer. Figure 1 illustrates the different organization of the three cyclases into domains.

Membranous Adenylyl Cyclase. Mammalian mACs consist of an N-terminal domain, two membrane-spanning domains (TM1, TM2), each with six

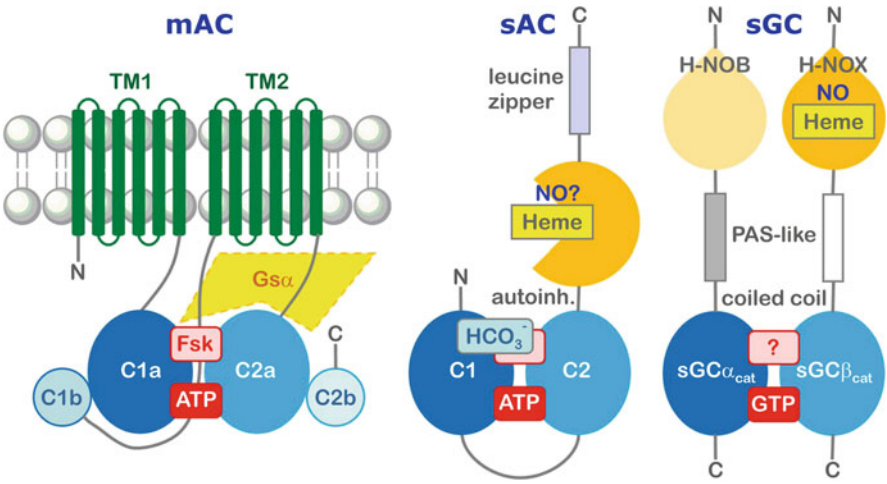

Fig. 1 Schematic representation of the overall domain structure and regulators of mAC, sAC, and sGC. For abbreviations, see text. © Stefan Dove, all rights reserved

transmembrane α-helices and two cytosolic cyclase domains. The latter are subdivided into catalytic (C1a, C2a) and regulatory (C1b, C2b) subdomains. C1a and C2a, the components resolved by X-ray structure analysis, show high structural homology among each other, among mAC isoforms, and with catalytic domains of sAC and sGC. The ATP binding site and a pseudosymmetric forskolin (Fsk) site are located between C1a and C2a. All nine mAC isoforms are activated by the G-protein $G_{s\alpha}$. Based on sequence similarities and additional modulators, they have been classified into four subgroups (Hanoune and Defer 2001; Patel et al. 2001): group 1 (isoforms 1, 3, 8), activation by Ca^{2+}/calmodulin; group 2 (isoforms 2, 4, 7), activation by $G_{\beta\gamma}$, insensitive to Ca^{2+}; group 3 (isoforms 5, 6), inhibition by Ca^{2+} and $G_{\alpha i/0}$, phosphorylation by protein kinases; and group 4 (isoform 9), insensitive to forskolin.

Soluble Adenylyl Cyclase. Mammalian sACs are more akin to bacterial sACs than to mammalian mACs and sGCs (Chen et al. 2000). Two splice variants exist, full-length sAC (157 kDa) and an alternative form just comprising the two catalytic domains (50 kDa, residues 1–490) (Jaiswal and Conti 2001).

Full-length sAC contains putative regulatory domains: an autoinhibitory stretch of 11 amino acids with unknown function (Chaloupka et al. 2006), a heme-binding domain (residues 897–1057) with CO and NO as hypothetical heme ligands (Middelhaufe et al. 2012) and a leucine zipper-like sequence (Buck et al. 1999). The catalytic domains C1 and C2 of sACs are directly regulated by bicarbonate, increasing turnover and relieving substrate inhibition, and by Ca^{2+} participating in ATP binding (Litvin et al. 2003). Binding sites of ATP, Ca^{2+}, and bicarbonate are located at the C1–C2 dimer interface (Kleinboelting et al. 2014a).

Soluble Guanylyl Cyclase. Mammalian sGCs are heterodimers of α- and β-subunits occurring in different isoforms, α1, α2, β1, and β2 (Mergia et al. 2003), among which α1 and β1 form the predominating dimer (Russwurm

and Koesling 2002). At the N-terminus, the β-subunit contains a heme-binding domain, H-NOX, whereas the corresponding domain of the α-subunit, H-NOB, does not bind heme. NO binding to the heme group in H-NOX leads to a conformational change strongly increasing catalytic activity of sGC (Ignarro 1991; Roy and Garthwaite 2006). The subsequent PAS-like domains have been suggested to trigger heterodimer formation of mammalian sGCs (Ma et al. 2008). Afterwards, in each subunit a putative coiled-coil bundle is followed by a catalytic domain, $sGC\alpha_{cat}$ and $sGC\beta_{cat}$, respectively (Allerston et al. 2013). High structural homology of $sGC\alpha_{cat}$ with C1 and $sGC\beta_{cat}$ with C2 of mammalian mACs and sACs indicates equivalent function. Also in case of sGC, the nucleotide binding site resides at the interface of the catalytic domains.

2 Overall Structure of Catalytic Domains

Sequence and 3D structure alignments of the catalytic domains of mACs, sAC, and sGC indicate that their high homology is mainly structure-based. In contrast, amino acid identities are only moderate. Nevertheless, comparison of only those sequences resolved in crystal structures reflects the close evolutionary relatedness of mAC and sGC catalytic domains, whereas sAC is quite distant from the other two enzymes (see Table 1). For each enzyme pair, the sequence identities between C1 ($C\alpha_{cat}$) and C2 ($C\beta_{cat}$) domains are very similar. Strikingly, the monomers $sGC\alpha_{cat}$ and $sGC\beta_{cat}$ are highly identical in contrast to VC1 and IIC2 and to the strongly different sAC-C1 and sAC-C2 domains.

Figure 2 shows a structure-based alignment of resolved sequences of the three nucleotidyl cyclases, considering a previous comparison of mACs and sGC (Tesmer et al. 1997), published when only crystal structures of VC1:IIC2 were known, and a recent alignment of all three enzymes including different prokaryotic and eukaryotic sAC species (Kleinboelting et al. 2014b). The general fold of VC1:IIC2 (Fig. 3) is very similar to that of $sGC\alpha_{cat}$:$sGC\beta_{cat}$ and, to a lesser extent, sAC-C1 and sAC-C2 (Fig. 4), in particular if only the sequences between β1 and α4 (β1′ and α4′, respectively) are regarded.

For each cyclase, the secondary structure elements in both catalytic domains are similar, too (Fig. 2). Superposition of VC1 and IIC2 results in a root-mean-square deviation of 1.3 Å for 153 structurally equivalent Cα atom pairs (Tesmer et al. 1997). In the complex, the domains form a head-to-tail, wreath-like dimer

Table 1 Amino acid identity within catalytic domains of nucleotidyl cyclases

	sGCβ	mAC IIC2	sAC-C2	
sGCα	**41.6%**	*27.0%*	*15.3%*	sGCβ
mAC VC1	28.9%	**25.3%**	*14.3%*	mAC IIC2
sAC-C1	16.5%	13.9%	**12.4%**	
	sGCα	mAC VC1		

Comparison of C1 ($C\alpha_{cat}$) domains, plain font, of C2 ($C\beta_{cat}$) domains, italics, of C1 with C2 ($C\alpha_{cat}$ with $C\beta_{cat}$) domains, bold

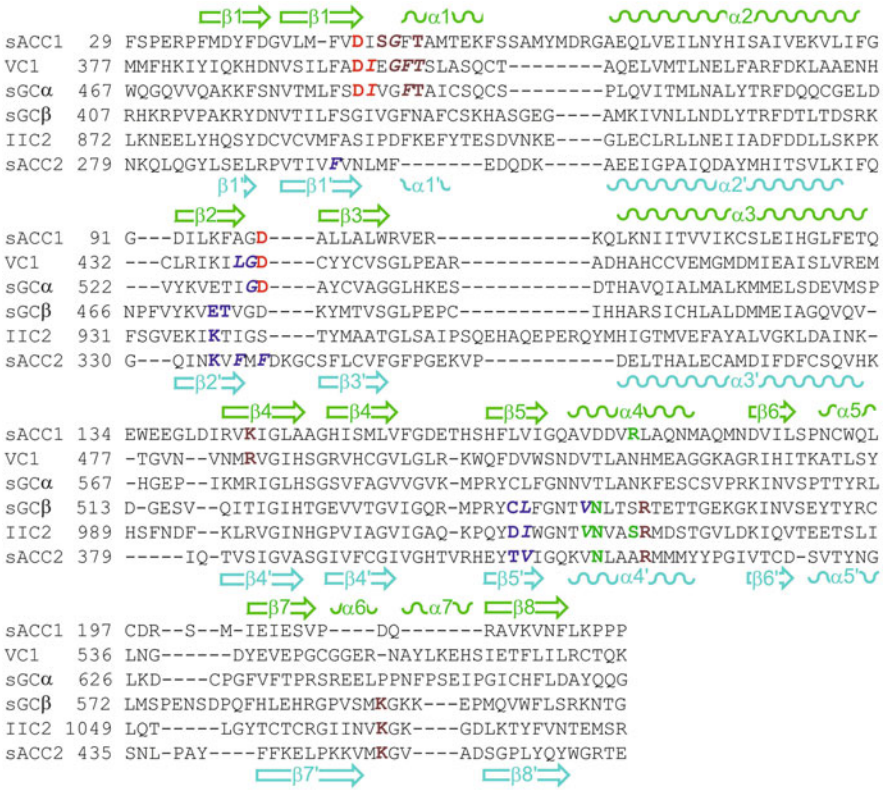

Fig. 2 Structure-based sequence alignment of the catalytic domains of sAC, mAC VC1:IIC2, and sGC. Secondary structure elements are primarily derived from VC1 (*green*) and IIC2 (*cyan*); after α5 of sAC-C1, VC1, and sGCα, only β7 and β8 are common. *Bold-colored amino acids* belong to the nucleotide binding sites and interact with metal ions (*red*), triphosphate (*brown*), the ribosyl moiety (*green*), and the nucleobase (*blue*), respectively. Amino acids in *italics* contribute via backbone, hydrophobic, or van der Waals interactions to nucleotide binding. Other interactions are electrostatic (including ionic, hydrogen bonds) and via side chains.

with a pseudo-twofold axis (Fig. 3), a topology common for mACs (Tesmer et al. 1997, 1999; Zhang et al. 1997), $sGC\alpha_{cat}$:$sGC\beta_{cat}$ (Allerston et al. 2013), and also for the C1-C2 association in the sAC monomer (Kleinboelting et al. 2014a). In both domains, five α-helices surround an eight-stranded β-sheet core in which, however, two strands (β1, β4 and β1′, β4′, respectively) are discontinuous. Some differences between the domains are eye-catching. The sequence alignment shows relatively variable α1–α2 and α1′–α2′ regions within and between the three structures. In case of VC1:IIC2, the lengths of α1 and α1′ as well as of the α1–α2 and α1′–α2′ loops differ in keeping with the function of the α1′–α2′ loop as part of the $G_{s\alpha}$ binding site which is additionally formed by α2′ and the α3′–β4′ loop. Helix α1′ is even not present in sAC-C2 (Fig. 4). The atypically long β3′–α3′ loop of IIC2 is not fully resolved in VC1:IIC2 structures and suggested to be involved in interactions with $G_{\beta\gamma}$ subunits (Chen et al. 1995). Striking differences between

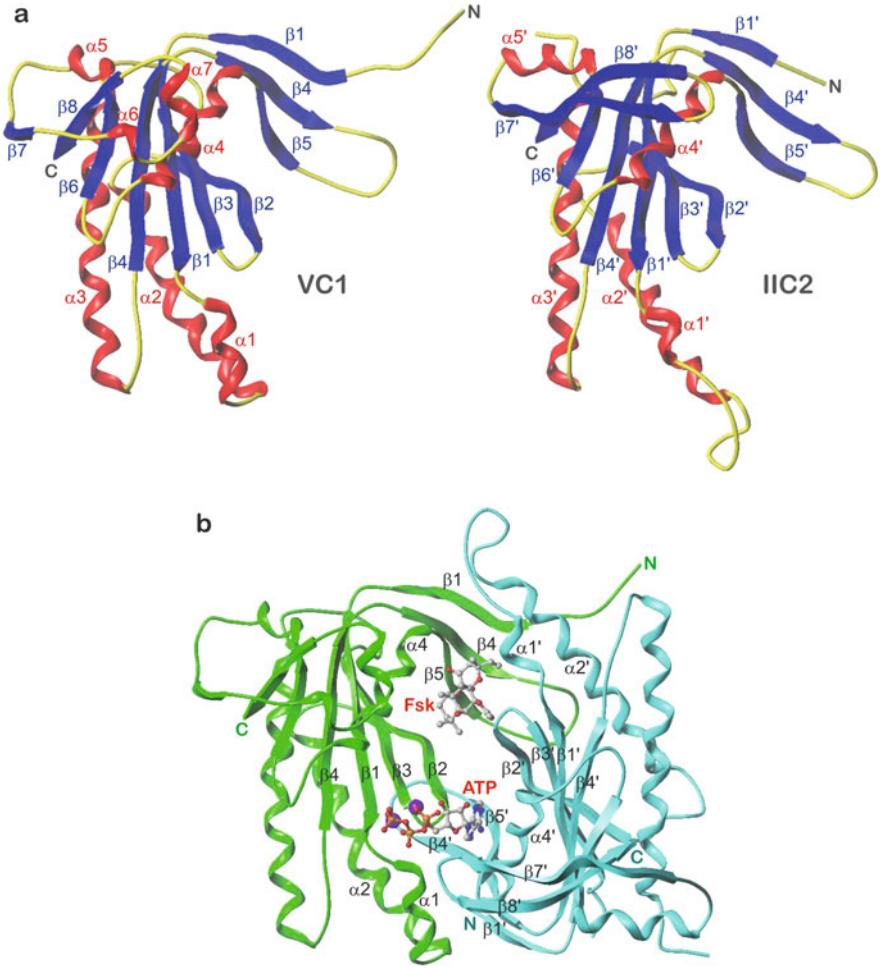

Fig. 3 (**a**) mAC VC1 and IIC2 in side-by-side comparison of superimposed monomers, α-helices, *red*; β-sheets, *blue*; loops and turns, *yellow*; (**b**) VC1:IIC2 in complex with forskolin and ATP, view along the pseudo-twofold axis; VC1, *green*; IIC2, *cyan*; atom colors C, *white*; N, *blue*; O, *red*; P, *orange*; Mg^{2+}, *violet*. Models are based on the crystal structure PDB 1CJU (Tesmer et al. 1997, 1999) with ATP docked instead of 2′,3′-dideoxy-ATP. Secondary structure elements (panel b only interface and binding sites) and N- and C-termini are labeled. © Stefan Dove, all rights reserved

the sequences and structures occur after α5 and α5′, respectively. VC1 contains a short β7-strand connected with β8 by a long Ω loop including two short α-helices which are lacking in $sGC\alpha_{cat}$ and sAC-C1. The course of $sGC\alpha_{cat}$ in this region is roughly similar to VC1. In sAC-C1, β7 is longer, and the course between β7 and β8 is different from VC1. The β7′–β8′ regions of the three structures are more

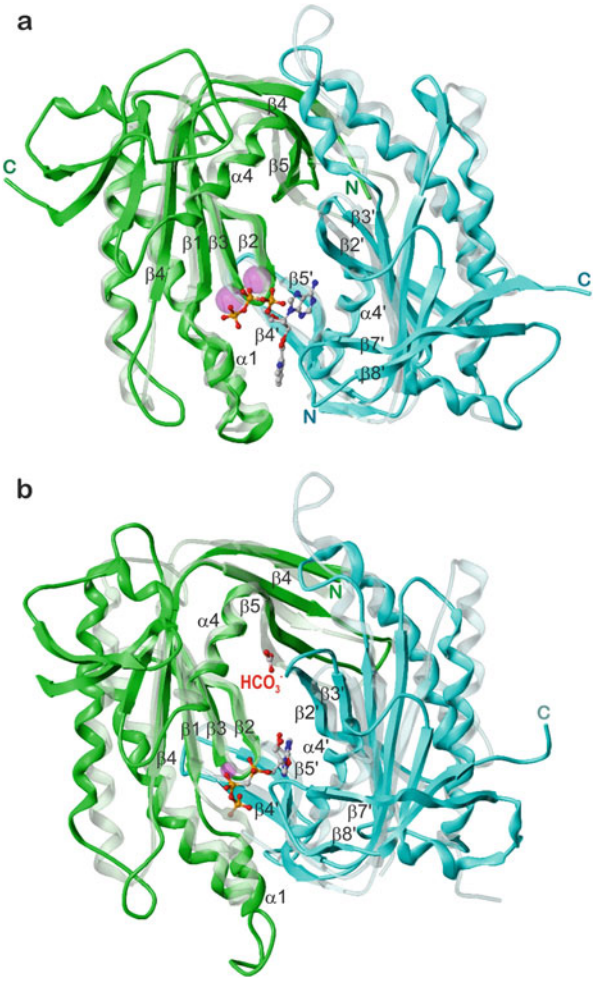

Fig. 4 Catalytic domains of sGC and sAC aligned with VC1:IIC2 (drawn from β1 to α4 and β1′ to α4′, respectively), views along the pseudo-twofold axes; $sGC\alpha_{cat}$, sAC-C1, VC1, *green*; $sGC\beta_{cat}$, sAC-C2, IIC2, *cyan*; atom colors C, *white*; N, *blue*; O, *red*; P, *orange*; Mn^{2+} and Ca^{2+}, *magenta*; secondary structure elements of the binding sites and N- and C-termini are labeled; (**a**) sGC_{cat} in complex with 2′-MANT-3′-dATP and Mn^{2+}, model of the intermediate state based on the open state crystal structure PDB 3UVJ (Allerston et al. 2013) and on VC1:IIC2 PDB 1TL7 (Mou et al. 2005) as template (transparent model); (**b**) sAC in complex with diphosphomethylphosphonic acid adenosyl ester, bicarbonate, and Ca^{2+}, model based on the crystal structure PDB 4CLK (Kleinboelting et al. 2014a), VC1:IIC2 (transparent model) corresponding to Fig. 3b.

similar, forming extended β-ribbons with a reverse turn participating in phosphate binding.

The interface of the catalytic domains is formed by two pseudosymmetric regions reflecting the pseudo-twofold symmetry of the whole complex. The more extensive upper region is characterized by contacts of the β4–β5 loop with β2′ and the β2′–β3′ loop, of α2′ with the N-terminus of β1 and with β4, as well as of the α1′–α2′ loop with the β5–α4 turn and β1. This region contains the binding sites of forskolin and bicarbonate in the case of VC1:IIC2 and sAC, respectively, and is not occupied in crystal structures of $sGC\alpha_{cat}$:$sGC\beta_{cat}$. Both forskolin and bicarbonate are suggested to stabilize domain interfaces and to contribute to the proper formation of the catalytic sites. The catalytic centers of the three cyclases with the nucleotide binding sites reside in the lower regions which also show the highest

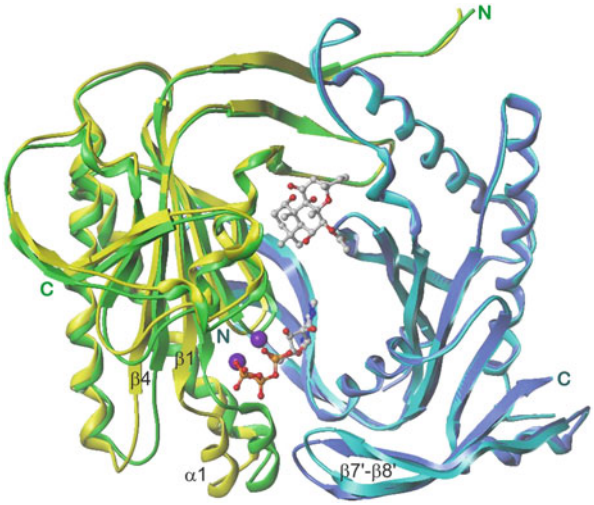

Fig. 5 Closed ATP-, Mg^{2+}-, and forskolin-bound conformation of VC1:IIC2 aligned with the open inactive state of apo-VC1:IIC2, view along the pseudo-twofold axes; closed state VC1, *green*; IIC2, *cyan*; open state VC1, *yellow*; IIC2, *purple*; atom colors C, *white*; N, *blue*; O, *red*; P, *orange*; Mg^{2+}, *violet*. Models are based on the crystal structures PDB 1CJU with ATP docked instead of 2′,3′-dideoxy-ATP and 1AZS, respectively (Tesmer et al. 1997, 1999). Different secondary structure elements and N- and C-termini are labeled.

identities within the sequence alignments. Compared to the pseudo-equivalent upper regions, significant differences become obvious.

Interactions of the β4′–β5′ loop with β2 and the β2–β3 loop as well as contacts of α2 with the β4′–β5′ loop and β1′ are weaker than opposite interactions in the upper region, and α1 does not essentially contact the second domain. Taken together, these differences allow high conformational flexibility of the lower pseudosymmetric region necessary for catalytic activity and its regulation.

In case of VC1:IIC2 and $sGC\alpha_{cat}$:$sGC\beta_{cat}$, ligand-free "open" and ligand-bound "closed" states have been distinguished. The catalytically active closed state results from a substrate-induced conformational change. Comparison of an apo-AC VC1:IIC2 structure with a 2′,3′-dideoxy-ATP bound one (Tesmer et al. 1997) indicated that translocation and 7° rotation of VC1 around the center of the heterodimer closes the catalytic site by moving α1 toward α4′ and the β7′–β8′ loop of IIC2 (Fig. 5).

A crystal structure of ATP-bound VC1:IIC2 with two inhibitory Ca^{2+} ions instead of Mg^{2+} represents an open state (Mou et al. 2009). By contrast, complexes of VC1:IIC2 with 2′(3′)-O-(N-methylanthraniloyl) (MANT) and 2′,3′-O-(2,4,6-trinitrophenyl) (TNP)-substituted nucleotides show intermediate (between closed and open) states due to binding of the substituents between α1 and the β7′–β8′ loop (Mou et al. 2005, 2006). A model of an active $sGC\alpha_{cat}$:$sGC\beta_{cat}$ state based on the crystal structures of the ligand-free heterodimer and the closed state of VC1:IIC2

indicated an even larger 26° rigid body rotation of the α-subunit to be necessary for GTP binding (Allerston et al. 2013).

3 Nucleotide Binding Sites

Unsurprisingly the nucleotide binding sites are the regions of highest similarity between mammalian mACs, sAC, and sGC. An evolutionarily well-conserved catalytic mechanism of cAMP and cGMP generation from ATP and GTP, respectively, as substrates is based on common binding modes, interactions, and structural transformations, including the participation of two metal ions in catalysis. Considering available crystal structures, some more or less substantial differences are due to substrate selectivity, adjustment and conformation of the ribosyl moiety, and flexibility of the triphosphate chain.

Figure 6 provides a schematic representation of the common nucleotide binding region and of positions of amino acids interacting with ATP and GTP, respectively. As already shown in the sequence alignment (Fig. 2), metal, triphosphate, ribosyl, and nucleobase interactions with the cyclases may be discriminated.

Metal ions A and B (Mn^{2+}, Mg^{2+}, Ca^{2+} or Na^{2+}) are coordinated with two aspartates located in β1 and the β2–β3 loop, respectively. Conserved basic residues (Arg, Lys) in β4, α4′ and the β7′–β8′ loop interact with the triphosphate chain so that each of the phosphates is involved. Additionally, the arginine in α4′ is essential for catalysis since it facilitates the protonation of the 3′-OH group. The ribosyl moiety

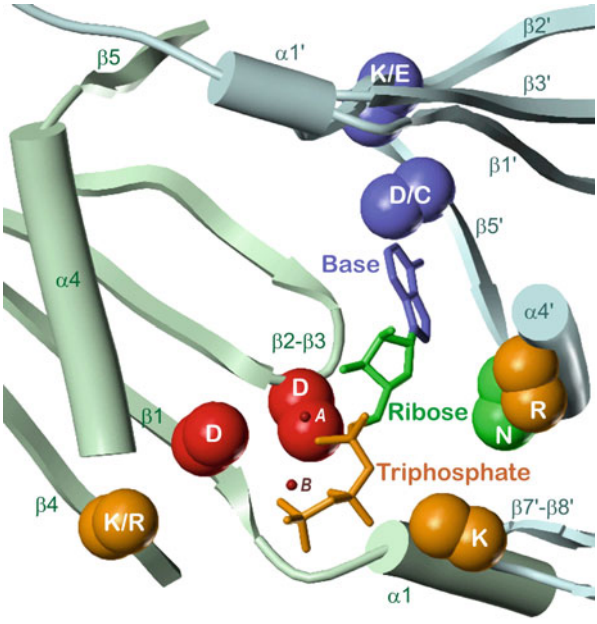

Fig. 6 Schematic representation of the common nucleotide binding site of mAC, sGC, and sAC. Essential amino acids are shown as space fill models of Cα and Cβ atoms; catalytic domain 1, *green*, catalytic domain 2, *cyan*, metals A and B, *red balls*, interactions of metal ions, *red*, triphosphate, *orange*, ribosyl moiety, *green*, nucleobase, *blue*. The model is based on the crystal structure PDB 1CJU (Tesmer et al. 1997, 1999) with ATP docked instead of 2′,3′-dideoxy-ATP. Common secondary structure elements and amino acids of the binding site are labeled.

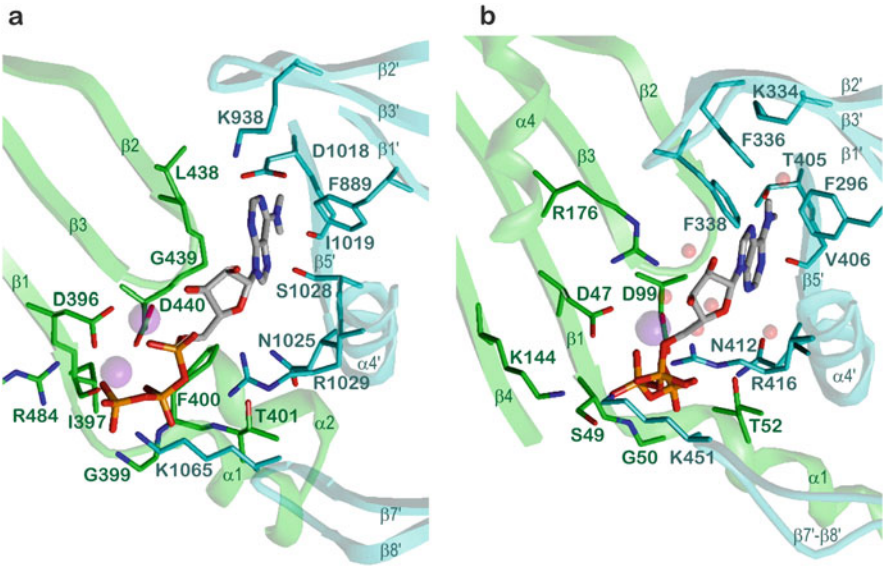

Fig. 7 Detailed binding modes of ATP at mAC VC1:IIC2 and sAC; model, labels, and C atoms of VC1 and sAC-C1, *green*; IIC2 and sAC-C2, *cyan*; other atom colors C, H, *white*; N, *blue*; O, *red*; P, *orange*; Mg^{2+}, Na^{+}, *violet*; (**a**) VC1:IIC2 in complex with ATP and Mg^{2+}, model based on the crystal structure PDB 1CJU (Tesmer et al. 1997, 1999) with ATP docked instead of 2′,3′-dideoxy-ATP; (**b**) sAC in complex with ATP, Na^{+}, and water (*pink balls*), model based on the crystal structure PDB 4USW (Kleinboelting et al. 2014b).

adopts varying ring conformations and binds in slightly different positions depending on substituents, the nature of the ligand (substrate or inhibitor), and the cyclase species. Residues in α4 and α4′ may contribute to ribosyl binding. Interactions with an asparagine in α4′ are common. The whole nucleoside moiety is embedded in a relatively spacious cavity bordered by the β2–β3 loop on one side, β5′ and α4′ on the other side, and by β2′ and the β2′-β3′ loop as roof. Nucleobase selectivity for ATP or GTP is mainly due to only two positions, namely, to a Lys-Glu exchange in β2′ and an Asp-Cys mutation in β5′ reversing adenine into guanine selectivity.

Figures 7 and 8 and Table 2 document detailed ligand-nucleotidyl cyclase interactions observed in four exemplary complexes (for localization of amino acids, compare Fig. 2). In Fig. 7a, ATP replaces the P-site inhibitor 2′,3′-dideoxy-ATP present in the VC1:IIC2 structure 1CJU. Both metal ions take part in catalysis (Tesmer et al. 1999). Serving as Lewis acid, metal A facilitates the nucleophilic attack of the 3′-hydroxy group on the α-phosphate and triggers the prior release of cAMP by displacement. The close distance between 3′-OH and metal A is due to a 3′-endo ribose puckering. Metal B participates in tight binding of the second product pyrophosphate which is also fixed by amino acids of a P-loop-like turn at the N-terminus of α1. Compared to sAC and sGC, binding of the base in *anti* orientation appears to be relatively strong due to three specific interactions (electrostatic, hydrogen bonds) of the adenylyl moiety.

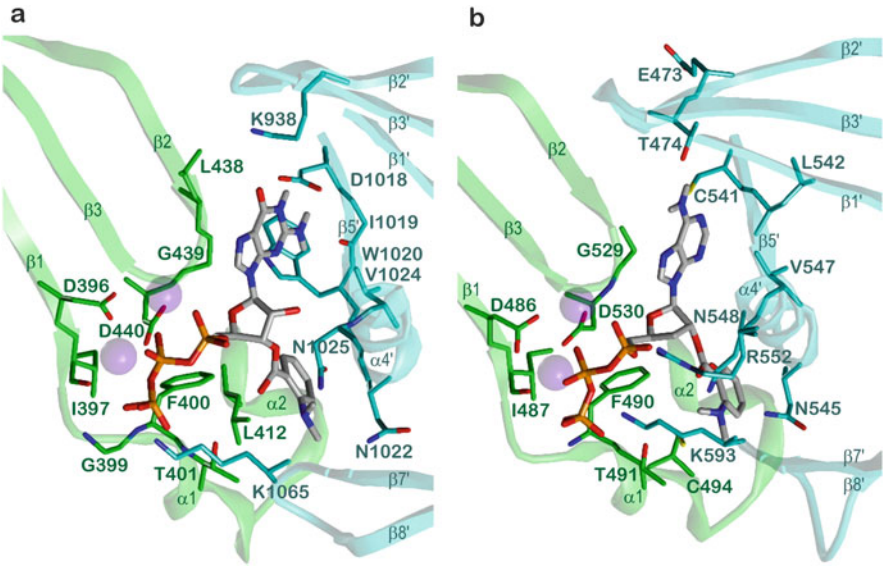

Fig. 8 Detailed binding modes of MANT-nucleotides at mAC VC1:IIC2 and sGC; model, labels and C atoms of VC1 and $sGC\alpha_{cat}$, *green*; IIC2 and $sGC\beta_{cat}$, *cyan*; other atom colors C, H, *white*; N, *blue*; O, *red*; P, *orange*; Mn^{2+}, *violet*; (**a**) VC1:IIC2 in complex with 3′-MANT-GTP and Mn^{2+}, model based on the crystal structure PDB 1TL7 (Mou et al. 2005); (**b**) $sGC\alpha_{cat}$:$sGC\beta_{cat}$ in complex with 2′-MANT-3′-dATP, model of the intermediate state based on the open state crystal structure PDB 3UVJ (Allerston et al. 2013) and on VC1:IIC2 PDB 1TL7 (Mou et al. 2005) as template.

In the sAC complex with ATP (Kleinboelting et al. 2014b), only one Na^+ (or Ca^{2+}) ion is located somewhat between the reference positions of metals A and B in VC1:IIC2, and six water molecules not present in the other three analyzed structures mediate sAC-ATP interactions (Fig. 7b). R176 actually points to the regulatory bicarbonate site but was modeled as in the sAC complex with α,-β-methylene-ATP (Kleinboelting et al. 2014a), suggesting that R176 together with R416 contributes to catalysis by facilitating deprotonation of the 3′-OH group. In contrast to almost all mammalian nucleotidyl cyclase structures resolved so far, the adenine base adopts a *syn* conformation. Additionally to the interactions listed in Table 2, base recognition is supported by a water-mediated interaction of the 6-NH_2 group with K334. It was suggested that a transient direct contact between K334 and the 6-NH_2 substituent is formed during catalysis (Kleinboelting et al. 2014a). Comparison of catalytic constants from ATP and GTP as sAC substrates has led to the conclusion that base discrimination takes place rather during turnover than by substrate binding (Kleinboelting et al. 2014b).

2′- and 3′-MANT-substituted NTPs bind to mAC similar as ATP (Hübner et al. 2011; Mou et al. 2005, 2006) as obvious in the complex of VC1:IIC2 with 3′-MANT-GTP (Fig. 8a). The ribosyl moiety adopts a 2′-endo conformation. An

Table 2 Detailed ligand-nucleotidyl cyclase interactions

	VC1:IIC2	VC1:IIC2	sGC[a]	sAC
Ligand	ATP[b]	3′-MANT-GTP	2′-MANT-3′-dATP	ATP
PDB	1CJU[c]	1TL7[d]	3UVJ[e] + 1TL7[d]	4USW[f]
Seq-Nr	VC1: 377–565; IIC2: 878–1,077	VC1: 377–565; IIC2: 879–1,077	α: 470–661; β: 412–607	C1: 29–216; C2: 279–467
Metal	MgA: D396, D440, α-*P*, 3′-OH; MgB: D396, D440, I397[g], α-, β-, γ-*P*	MnA: D396, D440, α-, β-*P*; MnB: D396, D440, I397[g], β-, γ-*P*	MnA: αD486, αD530, α-, β-*P*; MnB: αD486, αD530, αI487[g], β-, γ-*P*	NaB: D47, D99, β-, γ-*P*
Tri-*P*	α-*P*: R1029, MgA; β-*P*: F400[g], T401[g], MgB; γ-P: R484, G399[g], K1065, MgB	α-*P*: MnA; β-*P*: MnA, MnB; γ-*P*: K1065, G399[g], F400[g], T401[g], MgB	α-*P*: βR552, MnA; β-*P*: βK593, MnA, MnB; γ-*P*: αF490[g],αT491, βK593, MgB	α-*P*: R416, K451; β-*P*: S49, K144, NaB; γ-*P*: G50[g], T52, N412, NaB
Ribose	O: N1025; 2′-OH: S1028; 3′-OH: MgA	2′-OH: N1025[g]; *F400*, *V1024*	αD530[g], *αF490, βN548*	O: N412; 3′-OH: R416
Base	N_1: K938, NH_2: D1018, I1019[g]; *L438, G439, F889*	O_6: K938; N_1: D1018, NH_2: D1018, I1019[g]; *L438*, *G439*	NH_2: βT474; *αG529, βC541, βL542, βV547*	NH_2: T405, V406[g]; *F296, F336, F338*
MANT		*L412*, *W1020*, *G1021*, *N1022*	N: βN545, βN548; *αC494*, *βG544*	

Only direct interactions (not water mediated) are listed. Plain font: ionic and other electrostatic interactions, hydrogen bonds. *Italics*: van der Waals and hydrophobic contacts. *P* phosphate
[a]Homology model based on the inactive $sGC\alpha_{cat}$:$sGC\beta_{cat}$ structure and VC1:IIC2 in complex with 3′-MANT-GTP
[b]Replacing 2′,3′-dideoxy-ATP in the crystal structure
[c]Tesmer et al. (1999)
[d]Mou et al. (2005)
[e]Allerston et al. (2013)
[f]Kleinboelting et al. (2014b)
[g]Interaction with backbone N or O

axial orientation of the MANT group enables it to protrude between α1, α2 and the β5′–α4′ loop.

In this position, the MANT substituent behaves like a wedge and prevents the formation of the fully closed conformation (Mou et al. 2005). 2′-MANT and even 2′,3′-bis-MANT nucleotides may bind in a similar mode, but in these cases the ribose ring must adopt a 3′-endo conformation with an equatorial 3′- and an axial 2′-orientation (Geduhn et al. 2011). Intriguingly, the guanine base forms hydrogen bonds with the same amino acids (K938, D1018, I1019) as the adenylyl moiety in the VC1:IIC2-ATP complex (Fig. 7a). In both cases, the base adopts an *anti* conformation in contrast to the VC1:IIC2-3′-MANT-ATP complex with a *syn* position of the adenylyl moiety and with no specific enzyme-base interactions (Mou et al. 2006).

Alignment of the two sGC subunits $sGC\alpha_{cat}$ and $sGC\beta_{cat}$ (Allerston et al. 2013) with VC1 and IIC2, respectively, from the VC1:IIC2-3′-MANT-GTP complex (Mou et al. 2005) led to a model of a MANT-nucleotide binding, partially closed $sGC\alpha_{cat}$:$sGC\beta_{cat}$ conformation (Dove et al. 2014). Figure 8b shows this model docked with 2′-MANT-3′-dATP (most potent among analyzed MANT-nucleotides). Comparing Figs. 8a and b, the high similarity of binding modes and interactions is striking even if one takes into account that VC1:IIC2 served as template for $sGC\alpha_{cat}$:$sGC\beta_{cat}$. The axial orientation of the MANT substituent is due to a 3′-endo ribose pucker. As consequence, 2′- and 3′-MANT groups may occupy the same pocket. However, the aminomethyl group of 2′-MANT-3′-dATP seems to form two hydrogen bonds with N545 and N548 of $sGC\beta_{cat}$ which are not observed in the VC1:IIC2-3′-MANT-GTP complex. These additional unique interactions were suggested to account for the high potency of 2′-MANT-3′-dATP at sGC. The adenine base forms only one specific interaction, namely, a hydrogen bond of the 6-NH_2 group with the side chain of $sGC\beta_{cat}$ T474. The binding mode of the nucleobases of MANT nucleotides seems to be different from that of GTP. E473 and C541 from $sGC\beta_{cat}$ are necessary for GTP-guanine binding (suggested hydrogen bonds of C541 with O6 and of E473 with N1 and N2) (Allerston et al. 2013; Hurley 1998) but not in appropriate positions for similar interactions with 2′-MANT-3′-dATP. Interestingly, GC activity could be converted to AC activity by E473K and C541D mutation corresponding to VC1:IIC2 K938 and D1018, respectively (Sunahara et al. 1998; Tucker et al. 1998).

4 Promiscuity for Nucleobases

Up to now, crystal structures of mammalian adenylyl and guanylyl cyclases reflect promiscuity for nucleobases only in cases of VC1:IIC2 complexed with inhibitory purine nucleotides, namely, with 3′-MANT-GTP and 3′-MANT-ITP (Hübner et al. 2011; Mou et al. 2005). However, experimental data show that mACs, sAC, and sGC are promiscuous for binding of purine and pyrimidine nucleotides as well as their MANT and TNP derivatives. By contrast, catalytic activities are highly selective for the natural substrates ATP and GTP, respectively. Table 3 exemplarily lists data from enzyme assays.

Obviously, the affinity of MANT- and TNP-NTPs for one and the same mAC or sGC species varies by only 0.3–1 pK_i units with respect to the nucleobase. Different means and ranks of pK_i values for MANT and TNP derivatives as well as for cyclase species indicate slightly different binding modes. A singular value decomposition (SVD) analysis based on K_i values of 20 MANT- and TNP-nucleotides on VC1:IIC2 resulted in contributions κ_i from the independent structural components, namely, base, phosphate, and 2′(3′)-*O*-ribosyl substituent (Table 4) (Mou et al. 2006). The comparison of $p\kappa_i$ values may discriminate between more or less tight binding of the three components and of the individual chemical groups present in the 20 compounds (however, rather $p\kappa_i/3$ values represent additive affinity contributions to pK_i). The 2′- and 3′-MANT-ribosyl moieties contribute more to

Table 3 Inhibition constants and kinetic parameters of nucleotides and their MANT and TNP derivatives as substrates and inhibitors, respectively, of mACs, sAC, and sGC

			A	G	C	U
VC1:IIC2	MANT	pK_i[a]	7.80	7.74	8.04	8.21
	TNP	pK_i[b]	7.09	7.08	6.51	7.04
mAC2	MANT	pK_i[a]	6.48	6.21	6.16	6.34
	TNP	pK_i[c]	7.00	6.66	6.96	7.62
mAC5	MANT	pK_i[a]	7.00	7.28	6.82	7.49
	TNP	pK_i[c]	8.43	7.57	7.51	7.82
sGC	MANT	pK_i[d]	6.77	6.06	6.28	6.30
	TNP	pK_i[d]	7.67	7.97	7.01	7.29
	NTP as substrate	pK_m[e]	4.83	4.84	2.94	4.09
		v_{max}[e]	9.8%	100%	4.6%	18.8%
sAC	NTP as substrate	pK_m[f]	2.22	2.53	2.50	2.30
		v_{max}[f]	100%	0.32%	0.56%	0.65%

[a]Data from Pinto et al. (2011)
[b]Data from Mou et al. (2006)
[c]Data from Suryanarayana et al. (2009)
[d]Data from Dove et al. (2014)
[e]Data from Beste et al. (2012)
[f]Preliminary data (Wolter et. al., Inst. Pharmacol., Hannover Med. School, Germany)

Table 4 Contribution of structural components to pKi values of MANT- and TNP-nucleotides as inhibitors of mAC VC1:IIC2, based on an SVD of 20 compounds (Mou et al. 2006)

	Base						Rib. subst.		Phosphate				
	A	G	C	U	H	X	MANT	TNP	*P*	*PP*	*PPP*	*PPSP*	*PPNP*
$p\kappa_i$	5.54	4.31	3.76	3.96	5.34	0.61	11.85	8.52	0.24	3.35	7.22	6.35	6.32

H hypoxanthine, *X* xanthine, *Rib. subst.* ribosyl-MANT/TNP, *P* phosphate, *PP* diphosphate, *PPP* triphosphate, *PPSP* (γ-thio)-triphosphate, *PPNP* (β,γ-imido)-triphosphate

affinity than the TNP-ribosyl group. Whereas the α-phosphate is nearly without effect, addition of the β- and the γ-phosphate results in increasing affinity increments. Hence, the triphosphate group is optimal for affinity, and the contributions of the three phosphates are more than additive.

The promiscuity of VC1:IIC2 for nucleobases is reflected by the SVD analysis. Except xanthine, all bases contribute significantly to affinity but with lower increments than the MANT- and TNP-ribosyl as well as the triphosphate moieties.

Accordingly, mAC-nucleobase interactions are rather weak compared to the rest of the inhibitors, indicating flexible fit of the bases in a sufficiently spacious pocket as major cause of promiscuity. Nevertheless, if mean affinities of 20 inhibitors are considered instead of single pK_i values, purine bases (in particular adenine and hypoxanthine) lead to higher affinity than pyrimidine bases. However, the K_i values differ by less than one order of magnitude. Figure 9a exemplarily shows the putative binding mode of 3′-MANT-UTP at VC1:IIC2. The uracil moiety is docked

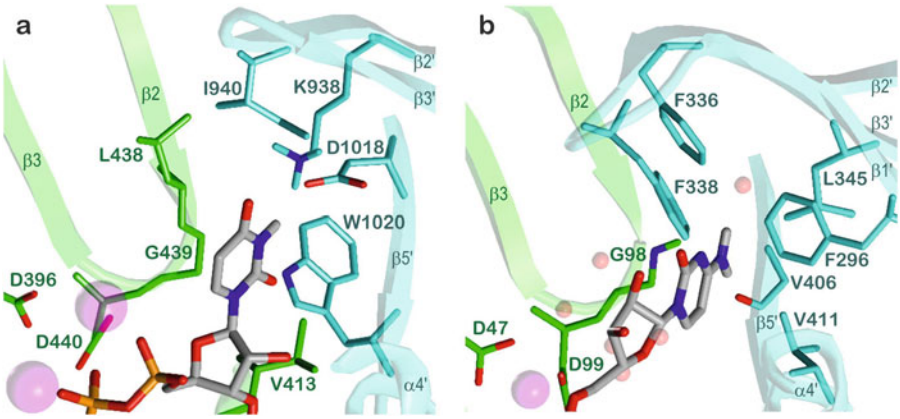

Fig. 9 Docking of pyrimidine nucleotides at mAC VC1:IIC2 and sAC; models, labels, and C and H atoms of VC1 and sAC1, *green*; IIC2 and sAC2, *cyan*; other atom colors C, H, *white*; N, *blue*; O, *red*; P, *orange*; Mn^{2+}, Na^{+}, *violet*; (**a**) VC1:IIC2 in complex with 3′-MANT-UTP and Mn^{2+}, model based on the crystal structure PDB 1TL7 (Mou et al. 2005); (**b**) sAC in complex with CTP, Na^{+}, and water (*pink balls*), model based on the crystal structure PDB 4USW (Kleinboelting et al. 2014b); views were pruned since triphosphate binding modes are the same as in Figs. 8a and 7b, respectively. © Stefan Dove, all rights reserved

in the same pocket as the guanine base in case of 3′-MANT-GTP (Fig. 8a). The O4 atom forms a hydrogen bond with the side chain of K938. A second, weaker hydrogen bond between the 3-NH function and D1018 is possible. van der Waals contacts with the backbone of L438 and G439 as well as with V413, I940, and W1020 are rather distant. At sGC, MANT-pyrimidine nucleotides can be similarly docked at the model shown in complex with 2′-MANT-3′dATP.

The binding mode of 3′-MANT-ITP at VC1:IIC2 (Hübner et al. 2011) suggests why hypoxanthine leads to high affinity in contrast to xanthine (Mou et al. 2006). Whereas the O6 atom and the 1-NH function of hypoxanthine interact with K938 and D1018 as in case of guanine (Fig. 8a), electrostatic repulsion between the O2 atom of xanthine and the backbone oxygen of I1019 prevents optimal fit.

The data in Table 3 indicate promiscuity for nucleobases also in case of sAC since pK_m values of ATP, GTP, CTP, and UTP differ by only 0.3. Replacing ATP by CTP in the recent sAC structure (Kleinboelting et al. 2014b) leads to the model in Fig. 9b. The cytosine moiety is embedded in the same hydrophobic pocket of the sAC-C2 domain as the adenine base of ATP (Fig. 7b), contacting F296, F336, F338, L345, and V411. Two weak hydrogen bonds of the O2 atom and the 4-NH_2 group with the backbone of G98 (NH) and V406 (O), respectively, contribute to CTP binding.

The catalytic activities of sGC and sAC (Table 3) demonstrate that other nucleotides than GTP and ATP, respectively, are poor substrates in spite of similar binding affinities (GTP, CTP, and UTP could never be shown to act as mAC substrates). Thus, discrimination of substrate nucleobases takes place during turnover and is not simply due to selective interactions in the Michaelis complex.

Optimal catalysis via a concerted mechanism probably requires specific flexibility of the base and some key residues. In case of sAC, coupling between ATP binding and turnover was suggested, including switch of the adenine orientation from *syn* in the Michaelis complex (Fig. 7b) into *anti* (Kleinboelting et al. 2014a, 2014b). In the transition state, a transient interaction of the N1 atom of adenine with K334 may be formed during ATP stretching and contributes to the separation of the α- and β-phosphate. K334 and T405 act as adenine-specificity mediating residues (Kleinboelting et al. 2014b).

The stable interaction of K938 with adenine in *anti* orientation in the VC1:IIC2-ATP complex does not rule out that the catalytic mechanism of mACs is similar since the model in Fig. 7a was based on the binding mode of the P-site inhibitor 2′,3′-dideoxy-ATP (Tesmer et al. 1997, 1999). Replacement of sAC-T405 by an aspartate (D1018 in VC1:IIC2) may be one of the reasons for the exceptionally high substrate specificity of mACs for ATP (Kleinboelting et al. 2014b). In contrast to threonine, aspartate is only a hydrogen bond acceptor which interacts with the 6-NH_2 group of adenine and not with the 6-oxygen of guanine.

In conclusion, the conditions that nucleotides are good substrates of nucleotidyl cyclases are strict. Nucleobase selectivity depends on specific stable and/or transient hydrogen bonds and on certain flexibility compatible with a concerted catalytic mechanism. Noncanonical nucleotides may be tightly bound but do not fulfill these conditions in detail because of at least partially different interactions. Thus, they are rather inhibitors but with significantly lower affinity than their MANT- and TNP derivatives.

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The *Pseudomonas aeruginosa Exoenzyme* Y: A Promiscuous Nucleotidyl Cyclase Edema Factor and Virulence Determinant

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Abstract

Exoenzyme Y (ExoY) was identified as a component of the *Pseudomonas aeruginosa* type 3 secretion system secretome in 1998. It is a common contributor to the arsenal of type 3 secretion system effectors, as it is present in approximately 90% of Pseudomonas isolates. ExoY has adenylyl cyclase activity that is dependent upon its association with a host cell cofactor. However, recent evidence indicates that ExoY is not just an adenylyl cyclase; rather, it is a promiscuous cyclase capable of generating purine and pyrimidine cyclic nucleotide monophosphates. ExoY's enzymatic activity causes a characteristic rounding of mammalian cells, due to microtubule breakdown. In endothelium, this cell rounding disrupts cell-to-cell junctions, leading to loss of barrier integrity and an increase in tissue edema. Microtubule breakdown seems to depend upon tau phosphorylation, where the elevation of cyclic nucleotide monophosphates activates protein kinases A and G and causes phosphorylation of endothelial microtubule associated protein tau. Phosphorylation is a stimulus for tau release from microtubules, leading to microtubule instability. Phosphorylated tau accumulates inside endothelium as a high molecular weight, oligomeric form, and is then released from the cell. Extracellular high molecular weight tau causes a transmissible cytotoxicity that significantly hinders cellular repair following infection. Thus, ExoY may contribute to bacterial virulence in at least two ways; first, by microtubule breakdown leading to loss of endothelial cell barrier integrity, and second, by promoting release of a high molecular weight tau cytotoxin that impairs cellular recovery following infection.

Keywords

cAMP compartmentalization • Nucleotidyl cyclase • *P. aeruginosa* virulence • Permeability • Tau toxicity

1 Introduction

Endothelium lines blood and lymphatic vessels throughout all organ systems, and in this capacity, serves as a semi-permeable barrier controlling the flux of solutes, macromolecules, and cells between the circulation and the underlying tissue (Townsley and Stevens 2015). This barrier property is highly dynamic, and is modulated according to the demands of the local environment. Adenosine 3′,5′-cyclic monophosphate (cAMP) is a ubiquitous second messenger that greatly influences the strength of endothelial cell adhesion (Sayner 2011). Circulating epinephrine (Sayner 2011) and prostacyclin (Birukova et al. 2007, 2012, 2013, 2015) activate transmembrane adenylyl cyclases, which increase endothelial cell cAMP that promotes cell-to-cell adhesion and decreases permeability (Fig. 1a). In contrast,

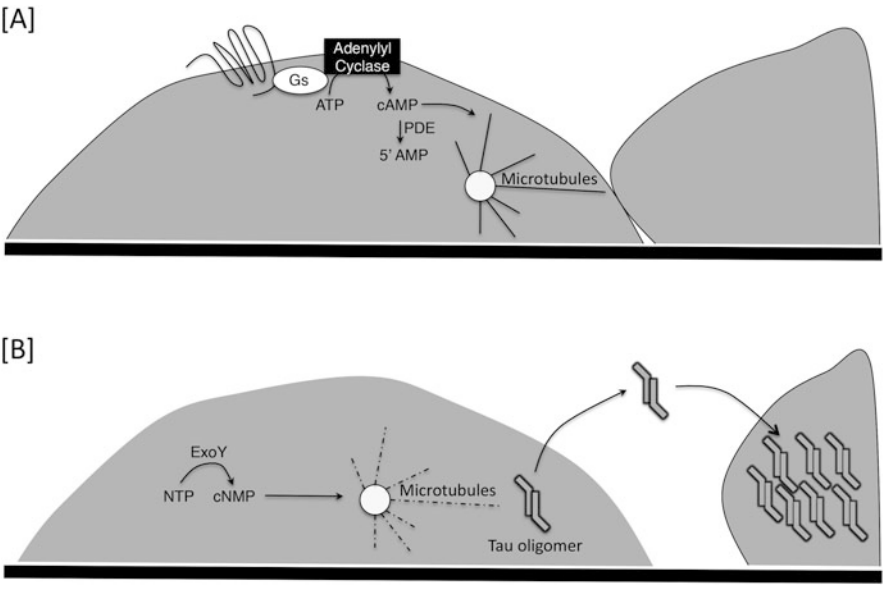

Fig. 1 Signaling through transmembrane and soluble adenylyl cyclases elicit different physiological outcomes in endothelium. (**a**) Circulating first messengers, such as epinephrine and prostacyclin, bind endothelial cell G protein coupled receptors that activate transmembrane adenylyl cyclases. Production of cAMP by transmembrane adenylyl cyclases strengthens the cortical actin rim, which stabilizes adherens junctions. This cAMP signal promotes microtubule interaction with peripheral actin. (**b**) ExoY is a promiscuous nucleotidyl cyclase that produces cGMP, cAMP, cUMP, and cCMP in endothelium. The cAMP signal is responsible for microtubule breakdown, leading to cell rounding and endothelial cell barrier disruption. cAMP, and to a lesser extent cGMP, activates PKA, which phosphorylates the microtubule associated protein tau. As tau becomes hyperphosphorylated, it is released from microtubules and forms apparent high molecular weight oligomers. These oligomers are released from the endothelium, and are transmissible among cells causing hyperpermeability and cytotoxicity. The stimulus for tau oligomer release is unknown. However, its release parallels the rise in cUMP, and emerging evidence suggests that cUMP may act as a second messenger important for stimulating tau oligomer release (unpublished). Other promiscuous nucleotidyl cyclases are used as part of the virulence arsenal of bacteria, including cyaA of *Bordetella pertussis*, edema factor of *Bacillus anthracis*, and MARTX of *Vibrio vulnificus*. These nucleotidyl cyclases have not yet been systematically studied in endothelium, although they are each soluble cylases with activity resembling ExoY

neurohumoral inflammatory calcium agonists inhibit endothelial transmembrane adenylyl cyclase activity, which decreases cAMP leading to loss of cell-to-cell adhesion and an increase in permeability (Cioffi et al. 2002; Stevens et al. 1995). Approaches to elevate endothelial cell cAMP concentration would therefore appear to represent an appropriate anti-inflammatory strategy (Sayner 2011; Moore et al. 1998). However, this is not the whole story.

Bacteria utilize soluble adenylyl cyclases as mammalian cell toxins, and these toxins contribute significantly to bacterial virulence (Sayner 2011). Such enzymatic activity was first described with the *Bacillus anthracis* edema factor (Leppla 1982) and *Bordetella pertussis* cyaA (Glaser et al. 1988), and more recently with the biotype 3 variant *Vibrio vulnificus* multifunctional autoprocessing RTX (MARTX)

toxin (Ziolo et al. 2014) and *Pseudomonas aeruginosa* exoenzyme Y (ExoY) (Yahr et al. 1998). Because *P. aeruginosa* is a principal cause of pneumonia that can progress to sepsis and acute lung injury, our group studied the contribution of ExoY to endothelial hyperpermeability (Fig. 1b). We have seen that ExoY functions not only as an adenylyl cyclase, but rather, as a promiscuous purine and pyrimidine nucleotidyl cyclase in vascular endothelium, generating cGMP, cAMP, cUMP and, to a lesser extent, cCMP (Morrow et al. 2015). The elevation of these cyclic nucleotide monophosphates causes endothelial tau hyperphosphorylation, which leads to microtubule breakdown and endothelial hyperpermeability (Balczon et al. 2013; Morrow et al. 2016; Ochoa et al. 2012; Prasain et al. 2009; Sayner et al. 2011). Hyperphosphorylation appears to trigger tau oligomerization and release, contributing to a transmissible proteinopathy that perpetuates cytotoxicity during infection (Morrow et al. 2016).

It is now apparent that cyclic nucleotide monophosphate signal transduction is far more complex than initially conceived. In endothelium, the location of cAMP production is a critical determinant of its end effect, where transmembrane adenylyl cyclases produce a cAMP signal that is barrier protective and cytosolic adenylyl cyclases produce a cAMP signal that is barrier disruptive (Sayner 2011; Moore et al. 1998). Studies exploiting the actions of ExoY have revealed that endothelium constitutively possesses both purine and pyrimidine (i.e., non-canonical) cyclic nucleotide monophosphates, yet little is known about the non-canonical cyclic nucleotide monophosphates. This chapter highlights key discoveries that led to our current understanding of cyclic nucleotide monophosphate signaling in endothelium, especially as it relates to control of barrier function.

2 The Endothelial cAMP Signal as a Barrier Protective Mechanism

Epinephrine has been a long-standing therapy for acute management of anaphylaxis. Among its many physiological effects, epinephrine binds endothelial β_2 receptors that are functionally coupled to an elevation in cAMP (Sayner 2011). This increase in intracellular cAMP activates protein kinase A (PKA), resulting in phosphorylation of effectors such as the actin binding protein filamin (Sayner et al. 2011). Phosphorylated filamin promotes F-actin alignment in a cortical rim that increases attachments among junctional complexes, strengthening the endothelial cell barrier. Thus, it is evident that cAMP arising from transmembrane adenylyl cyclases protects endothelial cell barrier integrity, and serves as a dominant intracellular signal controlling adhesion (Cioffi et al. 2002; Stevens et al. 1995).

It is curious then how circulating inflammatory agonists disrupt the endothelial cell barrier. Many of these agonists, such as substance P, histamine, bradykinin, and thrombin, elevate cytosolic calcium, which leads to myosin light chain kinase phosphorylation and realignment of the cortical actin rim into centripetally directed stress fibers (Dudek and Garcia 2001). This increase in tension is accompanied by loss of cell-to-cell adhesions, enabling formation of transient inter-endothelial cell

gaps that allow fluid, solute, and protein leak into the tissue (Mehta and Malik 2006).

The strength of this calcium signal is amplified by calcium inhibition of transmembrane adenylyl cyclases (Stevens et al. 1995). Endothelial cells express the type 6 adenylyl cyclase (AC6), which is inhibited by submicromolar increases in calcium, especially by calcium influx through store operated calcium entry channels (Cioffi et al. 2002; Creighton et al. 2003; Stevens et al. 1999). The sensitivity of calcium inhibition is most evident when measuring the ATP-to-cAMP turnover rates in plasma membrane fractions, although it can be detected by radioimmunoassay in whole cell lysates as well. Development of Fluorescence Resonance Energy Transfer (FRET) approaches to measure cAMP also enables visualization of AC6 calcium inhibition in single living cells (Werthmann et al. 2009). Depending upon the endothelial cell phenotype studied, the magnitude of calcium inhibition can range from 20 to nearly 90% of the membrane cyclase activity (Stevens et al. 1999), bringing into question the importance of this crosstalk mechanism in controlling endothelial cell barrier integrity.

To address this issue, a calcium *stimulated* adenylyl cyclase (AC8) was expressed in endothelium, and its impact on permeability assessed (Cioffi et al. 2002). AC8 was correctly targeted to the plasma membrane where it produced cAMP in apparent lipid raft domains, as appropriate. Expression of AC8 did not change baseline cAMP concentrations, yet it converted calcium inhibition into modest calcium stimulation of cAMP. Under these conditions, even high thrombin concentrations failed to disrupt the endothelial cell barrier, providing direct evidence that AC6 mediates physiologically relevant crosstalk between calcium and cAMP.

3 Edema Factor: A Soluble Adenylyl Cyclase

It seemed clear that elevations in cAMP protect the endothelial cell barrier, and further, that calcium influx lowers cAMP as a necessary prerequisite to the barrier disruption that causes edema. However, an apparent paradox to this understanding was lurking in the microbiology literature, and had not been considered by the vascular biology community. Studies in the 1950s from microbiologists examining *Bacillus anthracis* revealed that the death of infected animals was accompanied by tissue edema (Smith et al. 1955). Exudate obtained from an admixture of plasma and peritoneal and thoracic exudates was sufficient to cause small edematous lesions following intradermal injection. Thus, it appeared that *B. anthrax* produced some edema-causing agent; an agent that we would later learn is an adenylyl cyclase.

Over the ensuing 30 years, the molecular basis for anthrax toxicity was determined to depend upon three components: protective antigen, lethal factor, and edema factor (Keppie et al. 1963; Stanley and Smith 1961, 1963). Protective antigen binds to the mammalian cell surface to enable introduction of either lethal factor or edema factor. In the early 1980s, the molecular basis of edema factor came into focus (Leppla 1982). In what has become a citation classic, Leppla reported that edema factor increases mammalian cell cAMP. He considered the possibility that edema

factor increases cAMP because of the known actions of cholera toxin, which like edema factor, increases vascular permeability upon its introduction into the skin of animals (Craig 1965; Johnson et al. 1971). Cholera toxin induces ADP ribosylation of Gsα proteins, inhibiting GTPase catalytic activity, which allows transmembrane adenylyl cyclases to remain active, causing an increase in cAMP. Coincident with increased cAMP, cholera toxin also produces a characteristic CHO cell shape change. This change in cell shape could be reproduced using active edema factor, leading Leppla to consider edema factor was in some way generating intracellular cAMP. Leppla examined whether edema factor acts like cholera toxin, causing ADP ribosylation of a Gsα protein (referred to as ADP-ribosylation of the cyclase, since its Gsα target had not been determined). However, his evidence did not support this assertion, and rather, suggested that edema factor itself directly contributes to an adenylyl cyclase activity. We now know that this latter conclusion is correct. Edema factor is introduced into mammalian cells where it is localized within the cell's cytosolic fraction. In this fraction, edema factor interacts with calmodulin to confer enzymatic activity. As discussed below, evidence that edema factor activity is resolved within the cytosol is essential to the biological function of such "soluble" enzymes.

Edema factor is just one example of a broader family of soluble (class II) adenylyl cyclases (Linder and Schultz 2003). These enzymes are produced by multiple different bacterial species, including *B. anthracis* (e.g., edema factor), *Bordetella pertussis* (e.g., cyaA), *Vibrio vulnificus* (e.g., MARTX toxin), and *Pseudomonas aeruginosa* (e.g., ExoY). Our group became increasingly interested in the enzymatic activity of these soluble enzymes following discovery of ExoY. *P. aeruginosa* infection is a common cause of pneumonia that can progress to sepsis and acute lung injury, especially in immunocompromised patients. Moreover, *P. aeruginosa* pneumonia is described to cause vasculitis and coagulative necrosis, indicating interaction of the bacterium with the lung microvascular endothelium (Winn et al. 2008).

4 Studies on the Discovery of ExoY and Its Enzymatic Activity

Pseudomonas aeruginosa maintains a large genome that encodes the regulatory and structural genes required to infect a variety of hosts. Significant resources are dedicated to making use of a type 3 secretion system (T3SS), through which *P. aeruginosa* injects four known effector proteins (ExoS, ExoT, ExoU, and ExoY) (Engel and Balachandran 2009; Hauser 2009). Transcription of these four exoenzymes is regulated by the proximal transcription factor, ExsA. Prior to 1998, three of the four effector proteins had been identified and their activities during eukaryotic cellular infections described (Engel and Balachandran 2009; Hauser 2009). The functional roles of these effectors were postulated to facilitate infection of epithelial cells followed by dissemination of the bacterium to the bloodstream.

The fourth potential effector – now known to be ExoY – had been observed from early work comparing the secretome of ExsA(+) and ExsA(−) *P. aeruginosa* strains

(Yahr et al. 1997). The effector was identified as a 42-kDa protein that was detectable, albeit at much lower levels than ExoS and ExoT, in strains 388 and PAK but not in the extracellular supernatants of strain PA103. Amino-terminal sequences obtained in these analyses were subsequently used in a search of the newly sequenced (at that time) PAO1 genome (Stover et al. 2000). After adjusting for a frameshift and searching for the most probable open reading frame, a hypothetical protein corresponding to 42-kDa was identified that shared significant homology with the extracellular adenylyl cyclases of *Bordetella pertussis* (e.g., CyaA) and *Bacillus anthracis* (e.g., edema factor). The absence of signal sequence processing and possession of a consensus ExsA binding site suggested that this protein – ExoY – was a T3SS effector that may possess adenylyl cyclase activity. Yahr et al. (1998) determined that ExoY purified from *E. coli* possessed adenylyl cyclase activity and that amino acid residues important for ATP-binding were conserved in ExoY, CyaA, and edema factor. Although ExoY shares significant homology in the catalytic domain with CyaA and edema factor, the previously characterized calmodulin-binding domain was absent from ExoY. Calmodulin is required to measure edema factor catalytic activity, and it stimulates CyaA activity over 500-fold (Karst et al. 2010; Schuler et al. 2012; Selwa et al. 2012, 2014). ExoY, however, was not stimulated or activated by calmodulin in the Yahr studies (Yahr et al. 1998). Stimulation of adenylyl cyclase activity was, however, detectable after the addition of a post-nuclear extract from Chinese hamster ovary cells (CHO). This complex mixture of cytosolic proteins stimulated ExoY activity at least 500-fold in a dose–response relationship. Heated extracts were inactive, suggesting that the cofactor for ExoY was likely a protein. The identity of the eukaryotic cofactor(s) for ExoY remains elusive, although just recently, filamentous actin (F-actin) has been reported to be sufficient to confer ExoY activity (Belyy et al. 2016). To study the biological activity of ExoY, an effector-less strain of *P. aeruginosa* was constructed (PA103Δ*exoUexoT*::Tc) and rExoY was coordinately expressed with the T3SS machinery for injection into CHO cells (Yahr et al. 1998). In contrast to the biological activity of heat-labile enterotoxin and pertussis toxin, which caused an elongated or clustering of CHO cells, ExoY injected by the T3SS rounded CHO cells. This differential regulation of cell morphology was hypothesized to either be due to the magnitude of cAMP generation or to differences in subcellular distribution of the toxin. Two key studies by Sayner et al. confirmed the latter hypothesis. In the first of these studies, Sayner and colleagues utilized a *P. aeruginosa* strain (PA103Δ*exoUexoT*::Tc pUCP*exoY*) capable of introducing only ExoY into mammalian cells through the T3SS (Sayner et al. 2004). Using this bacterial strain, and a second strain introducing inactive ExoY (PA103Δ*exoUexoT*::Tc pUCP$exoY^{K81M}$), they determined that ExoY localized to the cytosol of pulmonary microvascular endothelial cells, and found that the ExoY-derived cAMP signal caused endothelial cell rounding leading to disruption of cell-to-cell adhesions and increased permeability (Sayner et al. 2004). This study provided direct evidence that ExoY acts like an "edema factor," directly contributing to permeability edema. The findings of this study were in stark contrast to the widely held belief that cAMP is barrier protective. However, they were taken to mean that the activation of soluble adenylyl cyclases, that is, enzymes located in

the cytosol, is barrier disruptive, whereas activation of transmembrane adenylyl cyclases is barrier protective.

In a second study, Sayner and colleagues (Sayner et al. 2006) evaluated whether transmembrane or soluble adenylyl cyclase activity dominates in control of endothelial barrier integrity. To test this idea, a soluble mammalian adenylyl cyclase chimera, the sACI/II enzyme, was expressed in endothelium. This chimeric enzyme mimics the localization of ExoY in the cytosol. The enzyme is constitutively inactive, and is directly stimulated by forskolin. Forskolin, however, also activates transmembrane adenylyl cyclases, and so it is capable of simultaneously producing cAMP from both transmembrane and soluble enzymes. In this case, forskolin acutely disrupted the endothelial cell barrier, demonstrating that soluble adenylyl cyclase activity dominates in control of the endothelial cell barrier (Sayner et al. 2006). An important facet of this work relates to the magnitude of increase in cAMP that is necessary to disrupt the endothelial barrier. sACI/II cytosolic activity is less than one-tenth of the transmembrane adenylyl cyclase activity, indicating that it takes very little cytosolic cAMP to disrupt the endothelial barrier. This issue is important to the effector function of ExoY, as ExoY will need to produce only low amounts of cAMP in order to cause edema.

5 ExoY and Disruption of the Endothelial Cytoskeleton

ExoS and ExoT possess RhoGAP activity, which remodels F-actin leading to cell rounding. Cowell and colleagues (Cowell et al. 2005) examined whether the adenylyl cyclase activity of ExoY was similarly associated with F-actin remodeling. $ExoY^{+}$ and $ExoY^{K81M}$ infections both led to apparent F-actin disruption in epithelial cells within 2-hours, suggesting the ExoY protein but not its enzymatic activity interferes with F-actin alignment. Just recently, the Mechold group (Belyy et al. 2016) revealed an interaction between ExoY and F-actin. F-actin was found to bind directly to ExoY where it served as a putative enzymatic cofactor. ExoY interaction with F-actin competitively dislodged other F-actin binding proteins, such as the Arp2/3 complex, providing a mechanism for remodeling the actin cytoskeleton. Consistent with these results, soluble adenylyl cyclase activity decreased phosphorylation of myosin light chain 20, indicating impaired actomyosin interaction. It therefore appears that ExoY intimately interacts with F-actin, perhaps necessary to confer its enzymatic activity, yet ExoY activity is not required for remodeling of the F-actin cytoskeleton.

Although ExoY intoxication does not promote F-actin contraction, it leads to progressive breakdown of peripheral microtubules, coincident with cell rounding, and inter-endothelial cell gap formation (Fig. 2). Balczon and colleagues examined the mechanism underlying this microtubule breakdown (Balczon et al. 2013). ExoY production of cAMP activates protein kinase A, which phosphorylates the microtubule associated protein tau. This endothelial cell tau stabilizes dynamic microtubule remodeling. Following protein kinase A phosphorylation, however, tau dissociates from microtubules, which promotes dynamic instability. Microtubule breakdown could result from decreased centrosome nucleation, increased rate of disassembly,

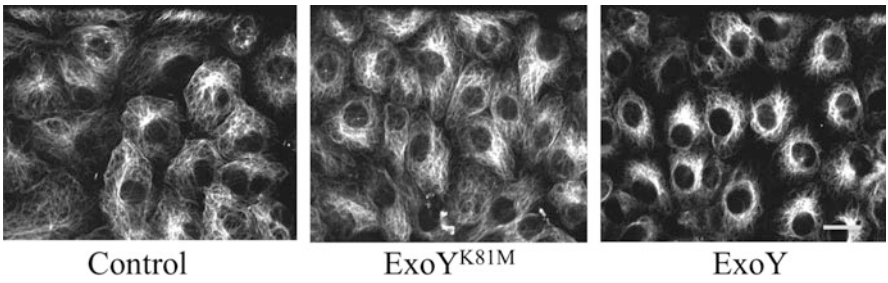

Fig. 2 Loss of peripheral microtubules following ExoY^{+} intoxication. Pulmonary microvascular endothelial cells were infected with either ExoY^{+} or ExoY^{K81M} at a multiplicity of infection (MOI) of 20:1, and microtubule abundance was assessed by anti-tubulin fluorescence microscopy. Microtubules were also assessed in uninfected (Control) cells. Scale bar = 10 μm. Adapted from Balczon et al. (2013)

or decreased rate of assembly. ExoY intoxication did not impact the rate of centrosome nucleation and it did not impact the rate at which cold exposure disassembled microtubules. Rather, ExoY decreased the rate at which microtubules assembled. Thus, ExoY catalytic activity targets endothelial cell tau for phosphorylation, causing microtubule breakdown by impairing its rate of assembly.

6 Discovery That ExoY Is a Guanylyl Cyclase

During the initial biochemical characterization of ExoY, GTP was added to a standard reaction mix to assess whether ExoY possessed guanylyl cyclase activity (Yahr et al. 1998). However, cGMP synthesis was not detected under these conditions and the assay for ExoY-mediated guanylyl cyclase activity was not revisited with assay conditions containing eukaryotic cytosolic cofactors. Thus, ExoY had been considered an adenylyl cyclase for approximately 12 years when Gottle et al. (2010) demonstrated that edema factor and CyaA could generate multiple cyclic nucleotides (namely cCMP, cUMP, and to a lesser extent cIMP (Gottle et al. 2010)). Given the shared enzymatic properties between ExoY, CyaA, and edema factor, it was reasonable to consider that ExoY generates multiple cyclic nucleotide monophosphates. Coupling this possibility with the fact that cAMP and cGMP both influence endothelial barrier integrity, Ochoa et al. (2012) hypothesized that ExoY possessed both adenylyl and guanylyl cyclase activity when introduced into pulmonary microvascular endothelial cells (PMVECs). When PMVECs were infected, ExoY intoxication generated a significantly higher (~10-fold) cGMP than cAMP signal, suggesting ExoY is principally a guanylyl cyclase (Ochoa et al. 2012). Yahr et al. (1998) previously demonstrated that substituting a single lysine residue within the ATP-binding domain at position 81 with methionine (e.g., ExoY^{K81M}) abolished adenylyl cyclase activity. ExoY^{K81M} also abolished ExoY's guanylyl cyclase activity. ExoY was coined a "promiscuous cyclase," as multiple lines of evidence now clearly illustrate ExoY's multifunctional enzymatic activity. The nature of the enzyme catalytic pocket enabling recognition of multiple

substrates has not yet been determined, although we consider that mammalian cofactor(s) may modulate substrate specificity, or alternatively, enzyme intracellular location may be a determinant of which substrate ExoY hydrolyzes.

7 ExoY as a Purine and Pyrimidine Cyclase

Given the structural similarities between *P. aeruginosa* ExoY, edema factor, and CyaA, coupled with evidence that CyaA and edema factor possess cytidylyl and uridylyl cyclase activity (Gottle et al. 2010), the possibility that ExoY might have broad substrate specificity, and therefore be able to generate purine and pyrimidine cyclic nucleotides, was reasonable. Beckert et al. (2014a) tested this idea in B103 neuroblastoma and A549 lung carcinoma cells. B103 cells were transfected with ExoY and purine and pyrimidine cyclic nucleotide monophosphates were measured by mass spectrometry in the same samples over a 3-day time course. They observed a long-lasting cyclic nucleotide monophosphate increase in the order cGMP ~ cUMP > cAMP ~ cCMP. Next, to evaluate the effects of a T3SS-delivered ExoY, they infected B103 and A549 cells at a multiplicity of infection (MOI) of 5:1 for 0–4 h and assessed cyclic nucleotide monophosphate levels. Four hours post-infection in B103 cells they observed a cyclic nucleotide monophosphate increase in the order cUMP ~ cGMP > > cCMP > cAMP. They observed a similar pattern in the A549 cells following infection. Interestingly, cUMP and cGMP increased first between 1–2 h post-infection, followed by increases in both cAMP and cCMP between 2 and 3 h post-infection. From these studies it is clear that ExoY: (1) generates both purine and pyrimidine cyclic nucleotide monophosphates in mammalian cells, and (2) its capacity to do so depends upon the cellular phenotype. To determine whether ExoY similarly increases purine and pyrimidine cyclic nucleotide monophosphates in vivo, Bahre et al. (2015) infected mice with *P. aeruginosa* ExoY$^+$. cUMP levels rapidly increased in lung tissue, and remained elevated 2–3 days post-infection. They also reported that cGMP levels exhibited a slow, gradual increase over a 24-hour time course post-infection (Bahre et al. 2015). The precise cNMP signal that is responsible for sustained vascular damage following ExoY$^+$ intoxication remains to be elucidated. Although these studies further supported the notion that ExoY is a promiscuous cyclase, the cell type (s) responsible for increased cyclic nucleotide monophosphates were unclear; it was suggested that both epithelial and endothelial cells contribute to this increase in cyclic nucleotide monophosphates. We examined whether ExoY$^+$ produces both purine and pyrimidine cyclic nucleotide monophosphates in lung endothelium (Morrow et al. 2015). Intoxication of pulmonary endothelium at an MOI of 20:1 with ExoY$^+$ produces cGMP first, followed by cUMP, and then cAMP; cCMP concentrations increase last, and this increase is of a relatively low magnitude. Although the temporal relationship among cyclic nucleotide monophosphates is similar between cell types, the magnitude of their increase is not. ExoY increases all cyclic nucleotide monophosphates to a greater extent in pulmonary artery than in pulmonary microvascular endothelial cells. This difference in cyclic nucleotide monophosphate metabolism has important implications regarding the physiological

response to infection, mechanisms regulating ExoY catalytic activity, e.g., enzyme cofactors, and potentially, cellular mechanisms responsible for cyclic nucleotide monophosphate turnover, e.g., phosphodiesterase activity and extrusion. Perhaps most provocatively, however, this work establishes a temporal relationship among cyclic nucleotide monophosphates as they are synthesized in endothelium, where cGMP is synthesized first, followed by cUMP and then cAMP. In future studies it will be important to resolve the signaling function of these cyclic nucleotides in time and space.

8 The Intracellular Function of Purine and Pyrimidine Cyclic Nucleotide Monophosphates

The function(s) of purine and pyrimidine cyclic nucleotide monophosphates in endothelium is complex and poorly understood. cGMP is sufficient to activate protein kinases G and A, which may contribute to tau phosphorylation. However, this cGMP signal does not appear to be the principal cause of tau phosphorylation (Ochoa et al. 2012). Other signaling roles played by cGMP in this context have not been explored, although the issue is important, as cGMP is not only the first, it is the most prominent signal produced by ExoY. cUMP increases second, along with cAMP. At present there is no known role for cUMP in endothelium. Evidence from the Seifert group (Wolter et al. 2011) indicates that cUMP can activate protein kinase A, and be hydrolyzed by phosphodiesterases 3a, 3b, and 9 (Reinecke et al. 2011), suggesting it serves as an important second messenger in mammalian cells. The cAMP signal best parallels endothelial cell barrier disruption, an effect that has been replicated by activation of other soluble adenylyl cyclases that produce only cAMP (Sayner et al. 2006). The ExoY-induced cAMP signal activates protein kinase A that clearly phosphorylates tau. Other intracellular targets of this cAMP response have not been thoroughly studied. Most of what we know about ExoY signaling is based upon its ability to increase cAMP and cause tau hyperphosphorylation.

Tau hyperphosphorylation has important physiological consequences, as this signal causes tau dissociation from microtubules leading to their breakdown. Tau accumulates in the endothelial cytosol for several hours following infection, and then is released in a high molecular weight, injurious form (Morrow et al. 2016) (see below). Interestingly, tau oligomerization and release closely parallels the increase in cUMP levels. Thus, it is possible that cUMP contributes to the release of oligomerized tau. This mechanism is of particular interest because, as has been recently established within the neurological field, extracellular tau oligomers are transmissible among cells as a mechanism of cytotoxicity (Frost et al. 2009; Kfoury et al. 2012).

9 Is ExoY Relevant to Disease?

Whether ExoY is relevant to the virulence of infection has been a topic of debate since its initial discovery in 1998. The definition of "virulence" varies among investigators, especially in light of the variety of cellular endpoints used to assess

Table 1 Commonly used *Pseudomonas aeruginosa* strains to assess virulence of type 3 secretion effectors

Strain	Isolation	T3SS effectors	Reference
PAO1	Wound	ExoS, ExoT, ExoY	Holloway (1955)
PA103	Sputum	ExoU, ExoT	Liu (1966a) and Liu (1966b)
PAK	Phage host	ExoS, ExoT, ExoY	Takeya and Amako (1966)
PA388	Burn wound	ExoS, ExoT, ExoY	Bjorn et al. (1979)
PA14	Burn wound	ExoU, ExoT, ExoY	Rahme et al. (1995)

it. Virulence is broadly defined as "the capability of a microorganism to cause disease." In the context of ExoY then, the relevant matter is whether or not introduction of ExoY through the T3SS contributes to pathology. The majority of studies addressing this issue have measured the: (1) severity of acute or initial infection, determined by host survival within the first 24–48 h post-infection; (2) bacterial dissemination or spread from the initial site of infection; (3) pathogen survival; or (4) cytotoxicity using in vitro culture systems. Multiple bacterial strains have been tested, including PAO1, PA14, PA103, and PAK (Bjorn et al. 1979; Holloway 1955; Liu 1966a, b; Rahme et al. 1995; Takeya and Amako 1966) (Table 1). The broad conclusion from this work has been that ExoY does not feature prominently in the virulence of infection. However, the role that ExoY plays in infection is more complex than initially appreciated.

10 Evidence That ExoY Does Not Contribute to Virulence

Early studies addressing the contribution of ExoY to infection severity uniformly concluded it played little role in virulence. For example, deletion of *exoY* from the PAK strain (Fig. 3) did not impact CHO cell cytotoxicity, as measured by LDH release (Lee et al. 2005), and similarly, *exoY* deletion did not impact CHO cell survival, as assessed by a cell plating efficiency assay. In the latter case, cells were infected at an MOI of 10 for 3 h and then trypsinized, serially diluted, and seeded in culture medium containing antibiotics. Under these conditions, CHO cell plating efficiency was similar after infection by PAKΔY and PAK wild-type strains. Bacterial survival and dissemination were also assessed using a mouse pneumonia model. Here, the deletion of *exoY* (PAKΔY strain) did not impact either bacterial survival in the lung or bacterial dissemination to the spleen and liver when compared to control strains. In separate studies, A549 cells were infected with individual clinical *P. aeruginosa* isolates at an MOI of ~80 for 3 h and LDH release measured, and J774 cells were infected at an MOI of ~200 for 6 h and apoptosis assessed. Each clinical isolate was instilled into the nares of BALB/c mice, animal survival was monitored over the course of seven days, and each strain's LD_{50} was determined. In the background of ExoU, ExoY did not further increase cytotoxicity, apoptosis-like cell death, or virulence (Schulert et al. 2003), as assessed by each strain's LD_{50}. Overall, these studies were taken to mean that ExoY plays a limited pathogenic role.

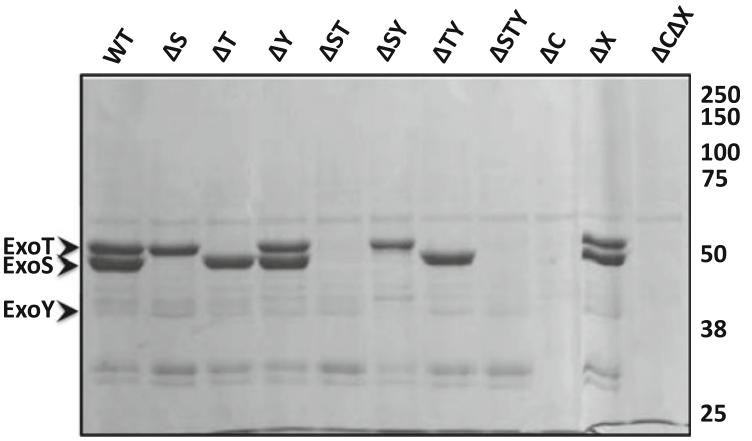

Fig. 3 Secretion profiles of *P. aeruginosa* strain PAK and isogenic derivatives induced for type 3 secretion. Secreted proteins were separated by 12% PAGE and revealed by staining with Coomassie. The positions of ExoS, ExoT, and ExoY on the gel are indicated by arrowheads. WT, wild type. Genetic deletion of *exoY* from PAK produces little to no change in ExoY protein levels. Adapted from Lee et al. (2005)

11 Evidence That ExoY Contributes to Virulence

Although early studies suggested that ExoY is functionally dispensable, emerging evidence reveals previously unappreciated ways in which ExoY contributes to virulence. ExoY initially causes cell rounding, due in part to a non-enzymatic regulation of the F-actin cytoskeleton and an enzymatic control of microtubule architecture. As cell rounding progresses to cellular death, the enzymatic activity of ExoY is responsible for production and release of amyloid proteins, like non-neuronal tau, that cause transmissible cytotoxicity.

The interaction between ExoY and the F-actin cytoskeleton remains poorly understood and represents an important area of study. Fleiszig and colleagues hypothesized that the adenylyl cyclase activity of ExoY may contribute to actin cytoskeleton disruption. As such, immortalized rabbit corneal epithelial cells were inoculated with either $ExoY^{+}$ or $ExoY^{K81M}$ at an MOI of 10, and F-actin microfilaments were observed by fluorescence microscopy 2 and 4 h post-infection. $ExoY^{+}$ and $ExoY^{K81M}$ both caused F-actin redistribution at 2 and 4 h. However, only cells infected with $ExoY^{+}$ displayed significant cell rounding, and this cell rounding was observed at 4 h (Cowell et al. 2005). These data are consistent with recent evidence implicating a role for F-actin as an ExoY cofactor, enabling production of cyclic nucleotide monophosphates that are responsible for cell rounding.

In a follow-up study, Hritonenko and colleagues hypothesized that ExoY may contribute to corneal epithelial cell virulence, an effect mediated by bacterial trafficking to plasma membrane blebs with subsequent bacterial survival and

replication. In cultured human corneal epithelial cells, ExoY adenylyl cyclase activity was sufficient to allow epithelial cell bleb-niche formation. However, whereas bleb-niche formation had previously been shown to correlate with bacterial survival and replication (Angus et al. 2010), there was no evidence that ExoY contributed to either survival or replication in vitro (Hritonenko et al. 2011). Interestingly, when applied to Black Swiss Surfactant Protein D knockout mice, $ExoY^{+}$ significantly increased the corneal disease severity score (used to assess virulence) at both 24 and 48 h post-inoculation, when compared with $ExoY^{K81M}$. Taken together, these results indicate that ExoY contributes to virulence in vivo without increasing bacterial survival or replication in vitro.

Beckert and colleagues determined that ExoY transfection into B103 neuroblastoma cells results in significant (~40% of total cell population) cell death 72 h post-transfection (Beckert et al. 2014a). This effect was hypothesized to be dependent upon cGMP and cUMP, due to ExoY nucleotidyl cyclase activity. As such, this group wanted to test whether the cytotoxic activity of ExoY was due to cGMP and cUMP generation. In a follow-up study, B103 cells treated with a combination of cGMP/cUMP-AM for 48 h displayed rounded cell morphology and apoptotic/necrotic cell death that was similar to cells transfected with ExoY (Beckert et al. 2014b). Although these studies were conducted in B103 neuroblastoma cells and were based upon a transfection approach rather than bacterial infection, they raise the possibility that ExoY in certain environments may do more than simply cause F-actin redistribution and cell rounding, as previously shown in CHO cells.

ExoY synthesizes cyclic nucleotide monophosphates; elevation of these cyclic nucleotide monophosphates results in phosphorylation of the microtubule associated protein tau. Tau hyperphosphorylation leads to its release from microtubules into the supernatant in an oligomeric form where it functions as a cytotoxic agent. Enrichment of purified oligomeric tau from cellular supernatant is sufficient to cause cell rounding and propagate injury in the absence of infection. Indeed, generation and release of oligomeric tau is an unequivocal mechanism of ExoY-induced virulence that has not been previously considered. The production of oligomeric tau is not just an in vitro phenomenon, but it is a part of the natural response to infection in vivo. $ExoY^{+}$ airway instillation causes pneumonia, which progresses to acute respiratory distress syndrome. Histological assessment of this infection reveals perivascular cuff formation and alveolar edema with hemorrhage (Stevens et al. 2014). $ExoY^{+}$ infection has long-standing consequences, as animals recovering from infection retain evidence for vascular dysfunction at least one week later, and preliminary studies reveal that oligomeric tau can be recovered from the bronchoalveolar lavage of infected animals 24 and 72 h post-infection (data not shown). Future studies will be required to assess the long-term impact of oligomeric tau exposure in the lung and determine the biodistribution of oligomeric tau among physiological compartments following infection.

There is no question that production and release of oligomeric tau contributes to ExoY-induced virulence. Interestingly, ExoU activity is also sufficient to induce oligomeric tau production and release. ExoU visibly damages endothelial cells within 3–4 h, whereas ExoY disrupts cell morphology within 6–8 h (Morrow

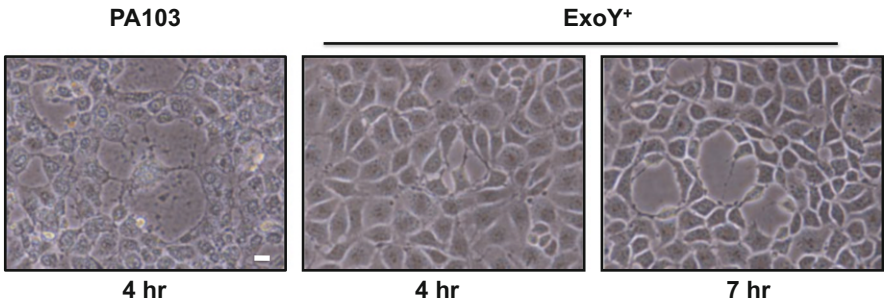

Fig. 4 Gap formation following *P. aeruginosa* infection. Pulmonary microvascular endothelial cells were inoculated with either PA103 or with ExoY$^+$ bacterial strain in Hanks' balanced salt solution (HBSS) at an MOI of 20:1, and gap formation was visually assessed by phase-contrast microscopy. Gap formation at 7 h post-infection with ExoY$^+$ was comparable to that observed at 4 h post-infection with PA103; overall cell health was worse in PA103-infected cells with prominent sunken nuclei observed. Images were captured at 20× magnification with scale bar = 10 μm. Adapted from Morrow et al. (2016)

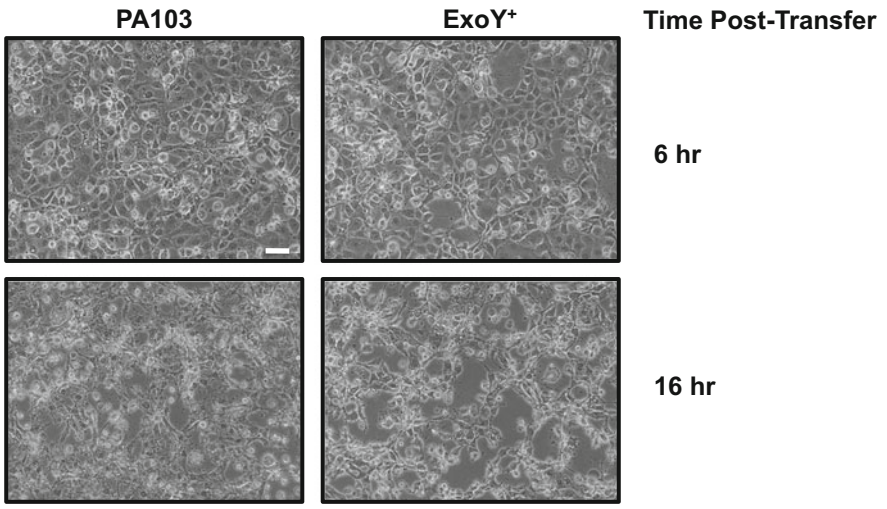

Fig. 5 Cytotoxic supernatant from PA103 and ExoY$^+$-infected cells applied to naïve endothelium. Filtered supernatants collected 4.5 h post-PA103 and 7 h post-ExoY$^+$ infection were transferred to naïve PMVECs, and images of injurious effects were captured 6 and 16 h post-transfer. Supernatant from ExoY$^+$-infected cells produced comparable damage to supernatant from PA103-infected cells. Images were captured at 20× magnification with scale bar = 10 μm. Adapted from Morrow et al. (2016)

et al. 2016) (Fig. 4). We have directly compared cytotoxicity due to ExoY$^+$- and PA103-generated supernatant. Supernatant collected from ExoY$^+$-infected cells causes cytotoxicity that is equal to or greater than supernatant collected from PA103-infected cells (Fig. 5). In addition, ExoY$^+$- and PA103-generated cytotoxic

supernatant containing high molecular weight tau causes endothelial barrier disruption in the isolated perfused lung. In considering the direct effect of ExoY on cellular morphology, its ability to cause necrosis, and its ability to generate cytotoxic tau oligomers, ExoY's contribution to bacterial virulence and pathogenesis is likely more extensive than initially thought.

12 Summary

Study of ExoY in vascular endothelium has led to a number of novel observations. Importantly, these experiments have given greater insight as to the importance of cAMP compartmentalization in control of endothelial cell barrier integrity, where cAMP generated by membrane adenylyl cyclases is barrier protective and cAMP generated by soluble adenylyl cyclases is barrier disruptive. The presence of pyrimidine cyclic nucleotide monophosphates in endothelium has now been reported. Further the intracellular milieu in pulmonary artery endothelial cells is more conducive to the production of all cyclic nucleotides by ExoY intoxication than is the environment in pulmonary microvascular endothelial cells. Evidence for expression of an endothelial tau isoform that stabilizes microtubules was obtained studying ExoY, and these observations led to discovery that ExoY enzymatic activity promotes tau oligomerization and release. In this latter case, tau oligomers are sufficient to cause cytotoxicity that perpetuates organ dysfunction even after the infection has been cleared. It appears increasingly evident that ExoY figures prominently among the arsenal of *P. aeruginosa* virulence factors. It will be important moving forward to address how the bacterium utilizes the complexity of T3SS effectors to interact with host cell microenvironment, especially among phenotypically diverse host cell populations.

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Cyclic Nucleotide Monophosphates in Plants and Plant Signaling

Claudius Marondedze, Aloysius Wong, Ludivine Thomas, Helen Irving, and Chris Gehring

Contents

Abstract

Cyclic nucleotide monophosphates (cNMPs) and the enzymes that can generate them are of increasing interest in the plant sciences. Arguably, the major recent advance came with the release of the complete *Arabidopsis thaliana* genome that has enabled the systematic search for adenylate (ACs) or guanylate cyclases (GCs) and did eventually lead to the discovery of a number of GCs in higher

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plants. Many of these proteins have complex domain architectures with AC or GC centers moonlighting within cytosolic kinase domains. Recent reports indicated the presence of not just the canonical cNMPs (i.e., cAMP and cGMP), but also the noncanonical cCMP, cUMP, cIMP, and cdTMP in plant tissues, and this raises several questions. Firstly, what are the functions of these cNMPs, and, secondly, which enzymes can convert the substrate triphosphates into the respective noncanonical cNMPs? The first question is addressed here by comparing the reactive oxygen species (ROS) response of cAMP and cGMP to that elicited by the noncanonical cCMP or cIMP. The results show that particularly cIMP can induce significant ROS production. To answer, at least in part, the second question, we have evaluated homology models of experimentally confirmed plant GCs probing the substrate specificity by molecular docking simulations to determine if they can conceivably catalytically convert substrates other than ATP or GTP. In summary, molecular modeling and substrate docking simulations can contribute to the evaluation of cyclases for noncanonical cyclic mononucleotides and thereby further our understanding of the molecular mechanism that underlie cNMP-dependent signaling in planta.

Keywords
Adenylate cyclase · *Arabidopsis thaliana* · cAMP · cGMP · Cyclic nucleotide monophosphates · Guanylate cyclase · Plant · Second messenger

1 A Brief History of Cyclic Mononucleotide Signaling in Plants

While there is a large body of literature on cNMP-dependent signaling in lower and higher eukaryotes, both addressing the question of biological functions and mechanisms of action, the acceptance that cNMPs play a role in plant signaling was delayed and not without controversy (Gehring 2010; Irving and Gehring 2013; Lemtiri-Chlieh et al. 2011; Newton et al. 1999). One reason for this is that the levels of cAMP and cGMP reported in plants appear to be generally lower than in animals or lower eukaryotes (for review, see Bolwell 1995; Gehring 2010; Newton and Smith 2004; Schaap 2005). Another and possibly more important reason for the reluctant acceptance of these signaling molecules in higher plants is that until quite recently, there was no genetic or molecular evidence of nucleotidyl cyclases (NCs) in higher plants. Additionally, homology searches with annotated adenylate cyclases (ACs) or guanylate cyclases (GCs) from lower or higher eukaryotes do not identify candidate cyclases in higher plants (Ludidi and Gehring 2003; Roelofs et al. 2001). The breakthrough came when a motif search of the *A. thaliana* genome based on conserved and functionally assigned amino acids in the catalytic center of annotated GCs (Liu et al. 1997; McCue et al. 2000; Zhang et al. 1997) from lower and higher eukaryotes returned a candidate that also contains the adjacent glycine-rich domain typical for GCs. In this molecule, termed AtGC1, the catalytic domain

is in the N-terminal part. AtGC1 contains the arginine or lysine that participates in hydrogen bonding with guanine and the cysteine that confers substrate specificity for GTP (Zhang et al. 1997). Since the discovery of the first GC in higher plants, and based on rational modification of the search motif together with site-directed mutagenesis, many more candidate GCs have been predicted (Wong and Gehring 2013a, b) and indeed tested for enzymatic activity. The recently experimentally confirmed GCs include a wall-associated kinase-like GC (AtWAKL10) (Meier et al. 2010), the brassinosteroid receptor (AtBRI1) (Kwezi et al. 2007), the Pep R1 receptor (AtPepR1) (Qi et al. 2010), and the phytosulfokine receptor (AtPSKR1) (Kwezi et al. 2011), which to date is the best studied. Phytosulfokines (PSKs) are sulfated pentapeptides that modulate plant growth and differentiation, as well as responses to biotic stresses (Igarashi et al. 2012; Mosher et al. 2013; Shen and Diener 2013). AtPSKR1 is a leucine-rich repeat receptor kinase with a functional GC catalytic center embedded within its cytoplasmic kinase domain (Irving et al. 2012; Kwezi et al. 2011). Overexpression of AtPSKR1 in Arabidopsis protoplasts causes an over 20-fold increase in endogenous cGMP levels, and this, together with ligand-specific cGMP transients (Kwezi et al. 2011), indicates that the receptor has GC activity in vivo and implies that cGMP has a role in PSK-dependent responses.

The first *bona fide* (Moutinho et al. 2001) AC was reported in the African blue lily (*Agapanthus umbellatus*) and shown to act on pollen tube growth and reorientation that in turn is a prerequisite for fertilization and subsequent seed formation (Moutinho et al. 2001). The authors also showed that growing tubes display a uniform distribution of cAMP with a resting concentration of approximately 100–150 nM and that forskolin and dideoxyadenosine, both modulators of ACs (Cochaux et al. 1982; Florio and Ross 1983), can alter cAMP resting levels. Since this initial report, the search for ACs has continued, and given that several candidate ACs have been predicted (Gehring 2010), we can expect to see in vitro and in planta test results from some of the candidates in the near future.

Interestingly, in the single-cellular green alga *Chlamydomonas reinhardtii*, there are more than 90 annotated ACs and GCs, and they come in over 20 different domain combinations where NCs combine with, for example, leucine-rich receptor kinases or proteases (Meier et al. 2007). Although these NCs have been annotated based only on sequence homology with NCs in animals or lower eukaryotes and not experimentally tested, it is likely that many of them function as cyclases.

There is increasing evidence that cNMPs have complex physiological roles in plants, both as modulators of single reactions and at the systems level. Given that in plants GCs are best established, we present here an overview of cGMP-dependent processes (Fig. 1) and foresee that noncanonical cNMPs share some of the targets or can substitute cGMP. GCs can be activated by ligands such as PSK (Kwezi et al. 2011) or nitric oxide (NO) (Domingos et al. 2015; Mulaudzi et al. 2011) and cause cGMP transients that in turn directly or indirectly affect downstream processes in different cellular compartments such as the cytosol, the chloroplast, the mitochondria, and the nucleus. In the cytosol, cGMP can modify plasma membrane H^+-ATPases (Suwastika and Gehring 1999) and net cation transport (Pharmawati

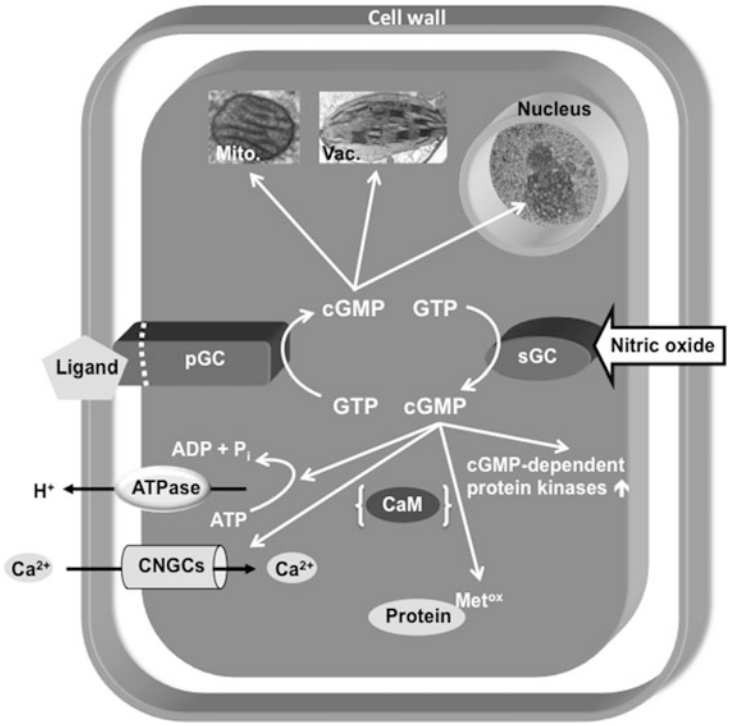

Fig. 1 Overview of cGMP-dependent processes in plant cells. Particulate (pGCs) or soluble GCs (sGCs) catalyze the reaction from GTP to cGMP that in turn modulates processes in the cell membrane, the cytosol, the mitochondria (Mito.), the chloroplast (Chlo.), or the nucleus. Proton transport is modulated by ATPases, while cation transport, including Ca^{2+} uptake, is enabled by cyclic nucleotide-gated channels (CNGCs) and calmodulin (CaM)

et al. 1999) including Ca^{2+} uptake (Ordoñez et al. 2014) conceivably through cyclic nucleotide-gated channels (CNGCs) (Talke et al. 2003; Zelman et al. 2012). Consistent with these responses is a cGMP-dependent accumulation of transcripts encoding monovalent cation transporters such as nonselective ion channels and cation/proton antiporters (Maathuis 2006). It has also been reported that cGMP is necessary and sufficient to transcriptionally induce a set of hormone (Bastian et al. 2010; Penson et al. 1996)-, ozone-, and nitric oxide-dependent (Pasqualini et al. 2009) genes. Furthermore, cGMP also causes distinct changes in the proteome signature (Ordoñez et al. 2014), in particular affecting proteins with a role in ion transport and stress responses and also resulting in specific posttranslational modifications such as phosphorylation (Facette et al. 2013; Yoshida et al. 2015) and methionine oxidation (Marondedze et al. 2013).

2 Evidence for Noncanonical Cyclic Nucleotide Monophosphates in Plants

The first systematic attempt to quantify the complement of cNMPs in plants was undertaken in the late 1980s (Newton et al. 1989). The authors report extraction of endogenous (3′,5′-) cAMP, cGMP, cCMP, cUMP, cIMP, and cyclic deoxyTMP (cdTMP) from meristematic and non-meristematic root tissue of *Pisum sativum* (Newton et al. 1989). Plant extracts were sequentially purified including an adsorption chromatography step on alumina, ion-exchange chromatography, and preparative electrophoresis. The purified samples were then compared to cyclic mononucleotide standards by HPLC and UV absorbance spectrophotometry, and further analysis was carried out by fast atom bombardment mass spectrometry and mass-analyzed ion kinetic energy spectrometry. The results indicated the presence of cAMP, cGMP, cCMP, and cUMP in meristematic tissue and cAMP, cGMP, cCMP, cIMP, and cdTMP in the non-meristematic tissues. The authors also report that there was a significantly higher concentration of cCMP in meristematic tissue as compared to non-meristematic tissues (Newton et al. 1989). The authors speculated that the elevated cCMP levels in the meristem might reflect a role in the rapidly dividing cells, analogous to that proposed for cCMP in mammalian cells, while the higher levels of cUMP in non-meristematic cells might be a reflection of the different rates of cell proliferation in the two types of tissue. However, the authors did not report the concentration of the respective nucleotides within the tissues, and the comparatively low sensitivity of the methods used at the time does not completely eliminate the possibility of artifacts.

In a recent survey of cAMP, cGMP, cCMP, and cUMP concentrations, it was found that *Arabidopsis thaliana* green leaf tissue does contain about 15 pmol mg^{-1} protein of cAMP and about 10 x less cGMP, while no cCMP was detected (Hartwig et al. 2014). Perhaps surprisingly, Arabidopsis leaves do contain 5.21 ± 2.11 pmol mg^{-1} protein of cUMP, which in effect makes the cUMP levels higher than those of cGMP. It has also been speculated that ancestral soluble ACs may be able to catalytically convert CTP and UTP to cCMP and cUMP, respectively (Hartwig et al. 2014; Seifert 2015).

3 Cyclic Mononucleotides and Their Role in Plant Reactive Oxygen Species (ROS) Signaling

In tobacco, defense gene induction has been shown to be critically dependent on ROS, notably nitric oxide (NO), as well as the messengers cGMP and cyclic ADP-ribose that function downstream of NO (Durner et al. 1998; Klessig et al. 2000). There is increasing evidence that cyclic nucleotides also have a role in the responses to ROS in Arabidopsis (Ordoñez et al. 2014). Biologically active ROS include superoxide and hydrogen peroxide (H_2O_2), and the superoxide and hydroxyl radicals are capable of inducing cellular oxidative damage. In plants, these compounds are produced in excess as a result of environmental stresses such

as drought, salinity, and low temperatures as well as biotic stress such as pathogens (Apel and Hirt 2004; Sharma et al. 2012). Production of ROS is confined to cellular compartments that have strong electron flow such as the mitochondria, chloroplasts, and peroxisomes (Choudhury et al. 2013). ROS-mediated oxidative stress has been shown as the main symptom of toxicity; however, plants have a coordinated antioxidant defense or ROS scavenging mechanisms to counteract both enzymatic and nonenzymatic effects of ROS in order to achieve redox homeostasis (Munne-Bosch et al. 2013). In plants, a hypersensitive response leads to apoptosis or programmed cell death where ROS can be involved. It has also been shown that in systemic acquired resistance (a type of plant immune response), ROS interacts with salicylic acid (SA) in SA signaling (O'Brien et al. 2012). Besides oxidative toxicity, ROS can impact positively at low cellular concentrations by acting as a second messenger (Mittler et al. 2004).

In order to assess whether noncanonical cNMPs could play a role in ROS-mediated signaling and oxidation-reduction-related processes, an *Arabidopsis thaliana* cell suspension culture was treated with 100 μM of either cAMP, 8-bromo-cAMP, cCMP, or cIMP in addition to a ROS inducer (pyocyanin), a ROS inhibitor (*N*-acetyl-L-cysteine), or H_2O_2 as controls. The results showed that ROS production was significantly induced within 30 minutes in cell suspension cultures treated with cIMP. This was comparable to the treatment with pyocyanin and consistently observed in four biological replicates of two independent experiments. In the first experiment, ROS production was measured by the total ROS detection kit (Enzo Life Sciences, Lausen, Switzerland), which directly monitors real-time reactive oxygen and/or nitrogen species production in living cells and detects superoxide (Fig. 2a). In the second experiment, an OxiSelect intracellular ROS assay was used to measure H_2O_2 production enabling the monitoring of hydroxyl and other ROS activities within cells (Fig. 2b). Neither cAMP nor the cell-permeant cAMP analog, 8-bromo-cAMP, significantly induced ROS production. This observation has some similarity to mammalian cells, where, for instance, cAMP stimulated a low ROS response in granulocytes from normal patients compared to samples from type 1 and type 2 diabetic patients (Isoni et al. 2009; Nogueira-Machado et al. 2006). We further examined whether the ROS production observed from the live cells follows the same trend when cells are lysed and observed a different trend in the cytosolic fraction. After cell lysis, cAMP induces rapid and significant ROS production in response to treatment. In addition, cIMP, cCMP, and 8-bromo-cAMP as well as cAMP induced ROS production within 30 minutes. The induction of ROS can be interpreted as a type of cellular signaling mechanism that is both cNMP dependent and specific. Similar to cGMP, cIMP is not an oxidizing agent, but it can directly or indirectly induce ROS production. Cyclic GMP has been shown to cause protein oxidation (Marondedze et al. 2013), a role cIMP could conceivably share. We therefore speculate that cNMPs, and in particular cGMP and the noncanonical cIMP, also play key signaling roles in plant cell oxidation-reduction processes. The roles of noncanonical cyclic nucleotides as second messengers in animal systems and their pharmacological implications have recently been reviewed (Beste and Seifert 2013; Seifert et al. 2015), and it appears that the roles and

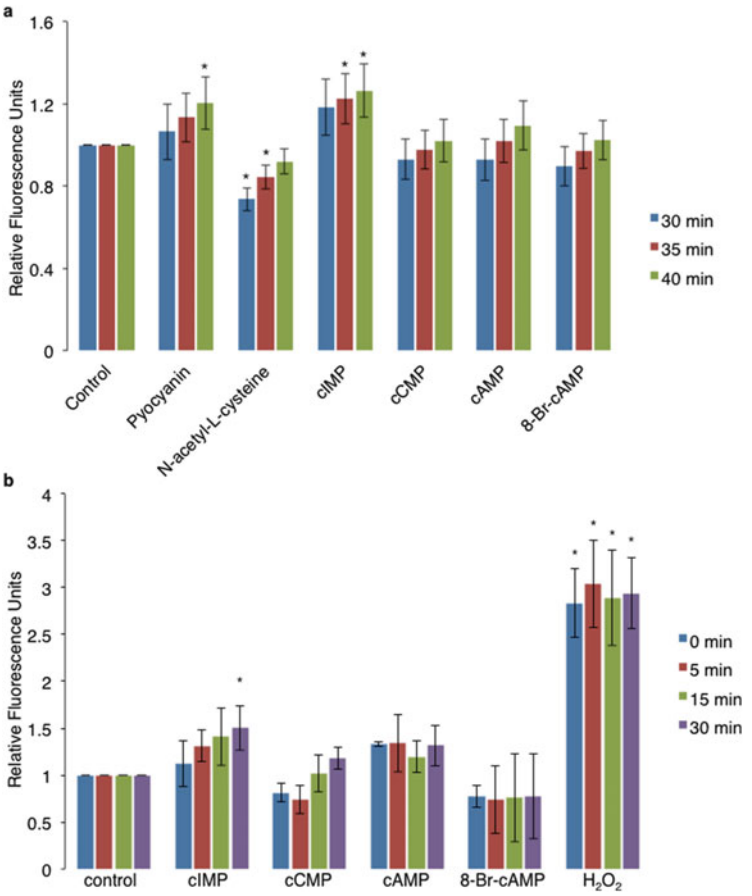

Fig. 2 *Arabidopsis thaliana* cell suspension culture oxidation assay. (**a**) Enzo ROS detection kit (Enzo Life Sciences, Farmingdale, NY) was used to assess in vivo levels of intracellular ROS generation following the assay protocol provided by the manufacturer. Cultured *Arabidopsis thaliana* (Col-0) cells were placed in a black bottom 96-well cell culture plate and a baseline reading was taken. Cells were loaded simultaneously with the treatment solution and the ROS detection solution. The treatment solution contains either positive control (ROS inducer (pyocyanin)), negative control (ROS inhibitor (*N*-acetyl-L-cysteine)), media solution (control untreated samples), or experimental samples containing a 10 μM of cIMP, cCMP, cAMP, or 8-Br-cAMP. Cells were stained for 30 min at 37°C in the dark, and fluorescence in the cells was measured at 30, 35, and 40 min posttreatment at 480/530 nm using a PHERAstar *FS* microplate reader (BMG Labtech GmbH, Germany) and the values plotted. *Each bar* represents data from five biological replicates ($n = 5$), and the *lines* are the standard errors. Treatment with cIMP at the final concentration of 10 μM induces statistically significant differences of the means at $p = 0.05$ using a two-sample *t*-test. (**b**) OxiSelect™ intracellular ROS assay kit (Cell Biolabs, Inc., San Diego, CA) was used in the in vivo oxidation experiments according to the assay protocol provided by the manufacturer. Cultured *Arabidopsis thaliana* (Col-0) cells were placed in a black bottom 96-well cell culture plate for 2 h in a shaking incubator. The 2′,7′-dichlorofluorescein diacetate/media solution was added to the cells prior to incubation for 1 h at 37°C. The dye-loaded cells were then treated with 10 μM of cIMP, cCMP, cAMP, 8-Br-cAMP, or H_2O_2. Fluorescence in the cells was measured at 0, 5, 15, and 30 min posttreatment at 480/530 nm using a PHERAstar *FS* microplate reader (BMG Labtech GmbH, Germany) and the values plotted. *Each bar* represents data from five

mechanisms of action of cIMP remain a source of debate (Seifert 2014). Recently and based on indirect evidence, it was proposed cIMP can be synthesized by soluble GCs and that this reaction mediates hypoxic contraction of coronary arteries (Chen et al. 2014).

4 Can Plant Mononucleotide Cyclases Conceivably Catalyze Noncanonical Nucleotide Triphosphates and Generate Their Corresponding Cyclic Nucleotide Monophosphates?

Given the reports of noncanonical cyclic mononucleotides in plants, we reviewed the catalytic center motif search and modeling approaches that have successfully identified functional GCs and predicted ACs in plants (Ludidi and Gehring 2003; Wong and Gehring 2013a, b) and discuss if the currently identified plant GCs can plausibly accommodate other nucleotide triphosphates (NTPs) and generate their corresponding cNMPs.

We have previously established that many plant GC centers share residence with larger primary domains at sites termed moonlighting centers (Freihat et al. 2014; Irving et al. 2012; Wong et al. 2015) and that these sites have important modulatory and/or signaling roles. They differ from, for example, the animal soluble and particulate GCs in that they are more likely to operate in localized spatial and temporal signaling events by acting as branch points (Freihat et al. 2014; Igarashi et al. 2012; Mosher et al. 2013; Zhou et al. 2013) that divert one pathway to another. In the phytosulfokine receptor (PSKR), the molecular switch is Ca^{2+} that can selectively shift the activation from the primary kinase to its embedded moonlighting GC (Muleya et al. 2014) that then generates cGMP to further inhibit the kinase (Kwezi et al. 2011). This intramolecular micro-regulation would therefore require transient levels of much lower amounts of cytosolic cGMP as compared to animal systems, and this is consistent with activities observed in plant GCs (Freihat et al. 2014). Several groups have detected cyclic mononucleotides in plant tissues (e.g., Donaldson et al. 2004; Hartwig et al. 2014; Isner et al. 2012; Maathuis 2006; Newton et al. 1999) and from in vitro enzymatic reaction of recombinant purified plant proteins (Kwezi et al. 2007, 2011; Ludidi and Gehring 2003; Meier et al. 2010; Mulaudzi et al. 2011; Qi et al. 2010), however at levels lower than those typically measured in animal GCs. Others failed to detect low levels of cyclic mononucleotides (Bojar et al. 2014), and this may be attributed to the lack of sensitive high-performance methods such as the liquid chromatography-mass spectrometry tandem (Beste et al. 2012; Hartwig et al. 2014). Detection by UV absorption of HPLC-separated fractions (Bojar et al. 2014) is not sensitive enough to

Fig. 2 (continued) biological replicates ($n = 5$), and the *lines* are the standard errors. Treatment with cIMP at the final concentration of 10 μM induces statistically significant differences of the means at $p = 0.05$ using a two-sample *t*-test

contrast cGMP from the background signal contributed by salts and cofactors that are present in the reaction mixture. Since a number of plant GC centers require specific conditions such as cofactors and dimerization for optimum catalysis, absence of these requirements, especially in in vitro assays, is one likely reason for low or lack of detection (Berkowitz et al. 2011; Freihat et al. 2014; Wheeler et al. 2013; Wong and Gehring 2013a). The PSKR1, when bound to its natural ligand alpha-phytosulfokine in mesophyll protoplast cells, has elevated GC activity (Kwezi et al. 2011), thus showing further evidence for an intricate transduction role that links ligand perception to the downstream cGMP-mediated cellular responses. Interestingly, the human interleukin 1 receptor-associated kinase 3 (IRAK3), uncovered using sequence homology-guided bioinformatic data-mining tools, was recently shown to generate cGMP at levels typical of plant GCs when expressed as a recombinant protein in both *E. coli* and human embryonic kidney (HEK)-293T cells (Freihat et al. 2014), thus implying that similar modulatory modes also exist in the animal system. This may now help to expand our current understanding of the signaling cascade downstream of the different GCs including the NO-dependent GCs (Domingos et al. 2015; Mulaudzi et al. 2011).

For the assessment of currently known plant GC centers, we used the well-characterized AtPSKR1 as a representative model since accurate homology models can be made based on the availability of highly similar template structures and docking simulations can be performed with confidence. The AtPSKR1 kinase homology model (Fig. 3a) accommodates a GC center that forms a distinct cavity at a separate region that does not overlap with the ATP binding site of the kinase domain. This GC center has an alpha helix fold that is followed immediately by a loop and accommodates the functional residues implicated in catalysis (Wong et al. 2015). Docking simulations implicate that GTP docked with the following binding pose: the hydrophobic nucleobase guanosine sits deep at the catalytic center and at distance close enough for establishing interactions important for catalysis with the experimentally determined functional residues at positions 1 and 3 (serine and glycine) at the GC center, while the negatively charged hydrophilic triphosphates point outward toward the solvent-exposed amino acid residue at position 14 (arginine or lysine) that has a positive net charge. In addition, this orientation also places the triphosphate end in the direction of the cofactor (Mg^{2+} or Mn^{2+}) that interacts with the amino acid (aspartic acid or glutamic acid) located two residues downstream of the GC center (Wong and Gehring 2013a; Wong et al. 2015). We define this substrate orientation as "suitable for catalysis." Further probing of the interacting residues at the catalytic center by site-directed mutagenesis and computational methods has been described previously (Wong and Gehring 2013a) and shown that the PSKR1 GC center, in the presence of docked GTP, displays structural and biochemical properties that are also present in other plant GCs identified to date.

Since NTPs all share very similar structural properties, it is conceivable that plant GCs can recognize them as substrates and are able to generate their corresponding cyclic mononucleotides. This proposition is supported by the fact that GCs and ACs in general poorly discriminate the different NTPs (Beste

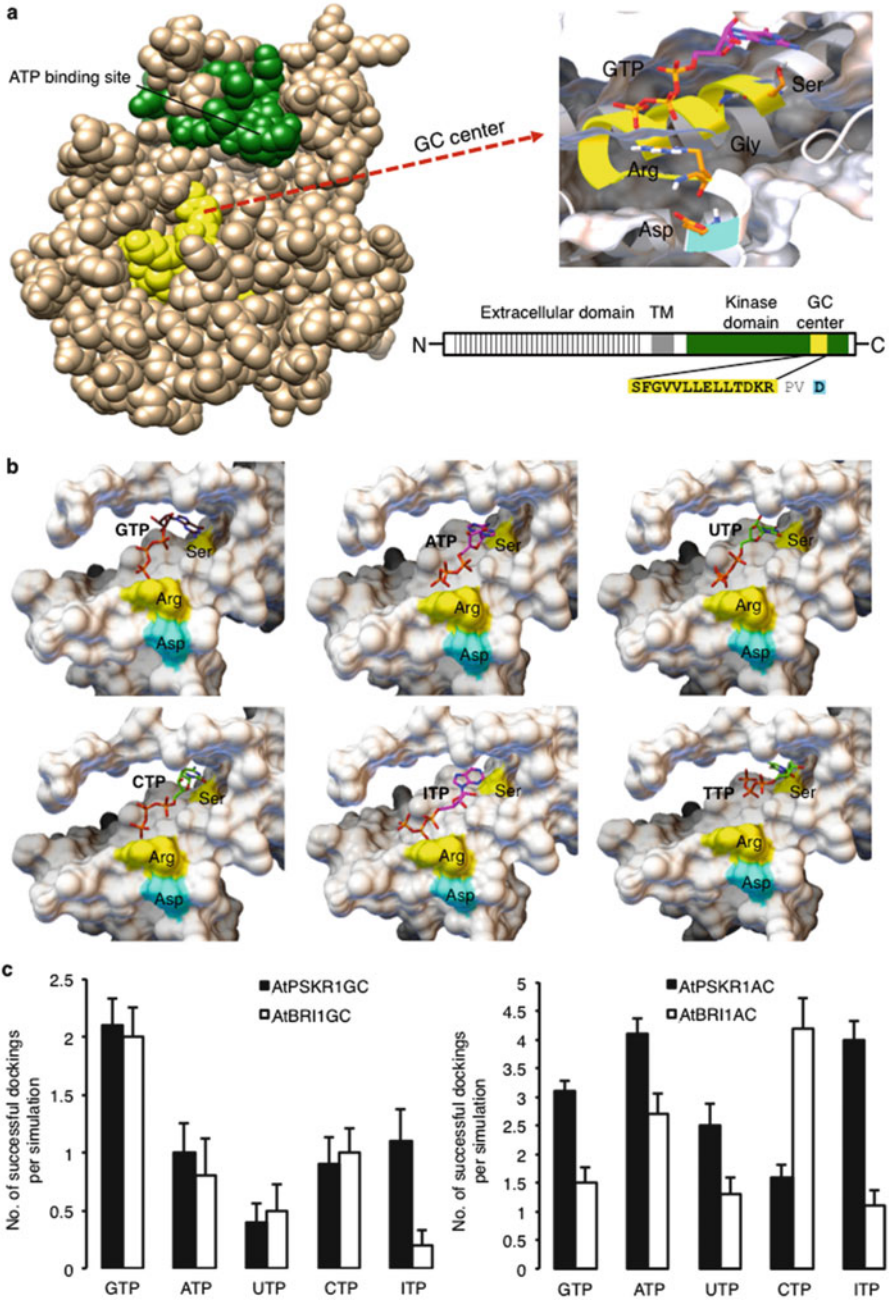

Fig. 3 Homology models of AtPSKR1GC and molecular docking of NTPs. (**a**) The homology model of AtPSKR1 kinase (Phe^{734}–Val^{1008}) and the domain organization of AtPSKR1 (Accession number: NP_178330.1) illustrate the ATP binding site (*green*) at the kinase domain and the GC center (*yellow*), and molecular docking of GTP to the GC center (*inset*) reveals the substrate pose and interactions with key residues at the GC center. Ribbons highlighted in *yellow* and *cyan* indicate the GC catalytic center and the metal-binding residue. (**b**) A representative model of

et al. 2012; Ruiz-Stewart et al. 2004; Sunahara et al. 1998; Surmeli et al. 2015). Perhaps remarkably, the retinal guanylyl cyclase (RetGC-1) can be converted into an AC by substitution of just two amino acid residues at the catalytic center (Tucker et al. 1998), while the *Dictyostelium discoideum* homolog of a mammalian soluble AC encodes a GC (Roelofs et al. 2001). Importantly, the recombinant purified rat soluble GC catalyzes GTP, ITP, XTP, and ATP and generates the corresponding cyclic mononucleotides in the presence of Mg^{2+}, but catalyzes only UTP and CTP when Mn^{2+} was provided in place of Mg^{2+} (Beste et al. 2012). Here, we performed in silico docking simulations using the NTPs: CTP, UTP, ITP, and TTP as substrates and the GC catalytic center of the model AtPSKR1 as the receiving molecule. When GTP was used as the ligand, successful docking that has favorable free binding energy and a binding pose deemed "suitable for catalysis" as previously defined occurred on average of over two times per simulation (Fig. 3c). As expected, the frequency of this binding pose was reduced to approximately one when ITP, ATP, CTP, and UTP were tested in place of GTP (Fig. 3c). A similar docking trend was also observed with the GC center of the AtBRI1, which has structure, domain architecture, and biological functions that are similar to that of PSKR1 (Fig. 3c). The binding poses of NTPs, in particular the noncanonical CTP, UTP, and ITP, satisfy the conditions for catalysis (Fig. 3b), although these solutions occurred less frequently compared to GTP (Fig. 3c). We note that binding *per se* is not diagnostic for catalysis; however, these simulations are useful for conducting initial screens to select plausible candidates and uncover structural insights as substrate binding is usually the defining rate-limiting step of enzymatic reactions (Wong and Gehring 2013a; Wong et al. 2015). Notably, only TTP did not yield any binding pose that can be deemed "suitable for catalysis" (Fig. 3b).

We further tested the substrate preference (or lack thereof) of AtPSKR1GC by replacing the residue at positions 1 and 3 of the catalytic center to arginine and glutamic acid, respectively, that is predicted to convert the GC into an AC. These mutations fit the derived AC motif that has been previously curated based on catalytic centers of ACs from animals and lower eukaryotes (Gehring 2010;

Fig. 3 (continued) AtPSKR1GC catalytic center (Asn^{871}–Glu^{980}) docked with the different NTPs. Amino acid residues that have catalytic functions are indicated in *yellow*, and the residue that is involved in metal binding is highlighted in *cyan*. All structures and images were prepared and analyzed with the UCSF Chimera – a visualization system (Pettersen et al. 2004). (**c**) Docking simulations of NTPs on the GC and the putative AC catalytic centers of AtPSKR1 and AtBRI1 (Accession numbers: NP_178330.1 and NP_195650.1). A total of ten docking simulations were performed, each generating nine solutions. The positive binding modes in each run were determined by analysis using PyMOL (ver 1.7.4) (The PyMOL Molecular Graphics System, Schrödinger, LLC), and the number of successful dockings per simulation were averaged. Homology models of AtPSKR1 (Phe^{734}–Val^{1008}), AtPSKR1GC (Asn^{871}–Glu^{980}), and AtBRI1GC (Leu^{1021}–Arg^{1134}) kinase were based on the AvrPtoB-BAK1 complex (PDB entry: 3TL8) which has a sequence similarity of 43% covering 99% of the queried amino acid sequence, and models were built and assessed using Modeller (ver. 9.10) (Sali and Blundell 1993). NTP docking simulations were performed using AutoDock Vina (ver. 1.1.2) (Trott and Olson 2010)

Wong et al. 2015) and did not cause structural changes at the catalytic center although the surface charge and the hydrophobic environment of the cavity were affected (Wong et al. 2015). Docking simulations were repeated using the putative AtPSKR1AC as the receiving molecule. Interestingly, docking of ATP with binding pose deemed "suitable for catalysis" occurred on average of over four times per simulation, while GTP was only three times (Fig. 3c). Both UTP and CTP have about 2.5 and 1.5 successful dockings (Fig. 3c), while ITP averaged four times. TTP did not yield binding poses that suggest suitability for catalysis (Fig. 3b). When the putative AtBRI1AC was used as the receiving molecule, GTP, UTP, and ITP have significantly lower docking frequency than that of ATP with the exception that CTP averaged a surprisingly high frequency of docking events (Fig. 3c). These mutations have resulted in a clear preference for ATP for the GC-derived AtPSKR1AC and AtBRI1AC over GTP (Fig. 3c), thus demonstrating that plant GCs like their animal counterparts have poor substrate discrimination (Beste et al. 2012).

The fact that in silico docking simulations afforded UTP, CTP, and ITP binding poses that are suitable for catalysis much like those generated from GTP and ATP dockings suggests that the identified plant GCs can rationally use these noncanonical NTPs as substrates and convert them into their respective cyclic forms. Therefore, highly sensitive experimental approaches should be used to investigate both in vitro and in vivo the capability of these known plant GCs to generate noncanonical cMNPs. If proven so, the molecular mechanism that assigns substrate specificity and affords substrate discrimination to these plant GC centers should then be elucidated. Some parallels can be however drawn from the animal systems, for example, the mammalian soluble GC has both allosteric and pseudosymmetric sites that accommodate other NTPs such as ATP, the binding of which modulates the GC activity (Surmeli et al. 2015). Alternatively, ions such as Mg^{2+} or Mn^{2+} can also dictate substrate specificities and affinities as evident in the catalytic activity of the rat soluble GC (Beste et al. 2012). It is also plausible that the sheer amounts of one NTP in relation to another can transiently, spatially, and/or temporally govern catalysis. Such regulations are vital when plant NCs, such as the modeled putative AC forms of AtPSKR1 and AtBRI1 that favors ITP and CTP to a degree similar if not greater than ATP (Fig. 3c), cannot discriminate these signaling molecules and are therefore consistent with the earlier described modulatory role of cyclic nucleotides (Freihat et al. 2014).

The detection of noncanonical cMNPs, in particular cUMP in the leaf of *Arabidopsis thaliana* (Hartwig et al. 2014), implies that cyclases capable of generating these molecules do exist in plants. Although we showed hints of cIMP being involved in ROS signaling in *Arabidopsis thaliana* suspension cultures and ITP can be rationally accommodated as a substrate for plant NCs, however, we note that cIMP has yet to be detected in plant tissues. Perhaps, the more relevant question is whether unique and designated proteins that exclusively generate cNMP molecules are present, and if so, do they exist as catalytic centers that are embedded within allosteric or moonlighting sites of larger multi-domain proteins much like in the currently characterized plant GCs? This will likely be answered as their

physiological significance, cellular signaling roles, and roles in hormone, biotic, and abiotic responses unfold in the near future.

5 Concluding Remarks

With the presence of noncanonical cNMPs established in plants, the quest for both their generation and biological function can be undertaken. A number of specific questions await answers. Firstly, can currently described and experimentally tested plant ACs and GCs catalyze substrates other than cAMP and cGMP, and, secondly, are there dedicated NCs with substrate specificity or preference for, e.g., UTP given that the cUMP levels in plants appear to be higher than those of cGMP. Furthermore, the biological roles of noncanonical cNMPs await elucidation, in particular the following questions: (1) Can noncanonical cNMPs modulate the gating of CNGCs (Zelman et al. 2012), and if so, how does their efficiency compare to cAMP or cGMP? (2) What do noncanonical cNMPs do to cAMP- and cGMP-dependent kinases? (3) Given that cGMP is necessary and sufficient to transcriptionally induce a set of hormone (Bastian et al. 2010; Penson et al. 1996)-, ozone-, and nitric oxide-dependent (Pasqualini et al. 2009) genes, will this also be observed in response to noncanonical cNMPs? (4) Can noncanonical cNMPs induce specific posttranslational modifications such as phosphorylation (Facette et al. 2013; Yoshida et al. 2015) and methionine oxidation (Marondedze et al. 2013)?

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cAMP-Dependent Protein Kinase and cGMP-Dependent Protein Kinase as Cyclic Nucleotide Effectors

Robin Lorenz, Daniela Bertinetti, and Friedrich W. Herberg

Contents

Abstract

The cAMP-dependent protein kinase (PKA) and the cGMP-dependent protein kinase (PKG) are homologous enzymes with different binding and activation specificities for cyclic nucleotides. Both enzymes harbor conserved cyclic nucleotide-binding (CNB) domains. Differences in amino acid composition of these CNB domains mediate cyclic nucleotide selectivity in PKA and PKG, respectively. Recently, the presence of the noncanonical cyclic nucleotides cCMP and cUMP in eukaryotic cells has been proven, while the existence of cellular cIMP and cXMP remains unclear. It was shown that the main effectors of cyclic nucleotide signaling, PKA and PKG, can be activated by each of these noncanonical cyclic nucleotides. With unique effector proteins still missing,

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such cross-activation effects might have physiological relevance. Therefore, we approach PKA and PKG as cyclic nucleotide effectors in this chapter. The focus of this chapter is the general cyclic nucleotide-binding properties of both kinases as well as the selectivity for cAMP or cGMP, respectively. Furthermore, we discuss the binding affinities and activation potencies of noncanonical cyclic nucleotides.

Keywords
cAMP • cCMP • cGMP • cIMP • CNB domain • cUMP • cXMP • Cyclic nucleotides • PKA • PKG • Protein kinases • Selectivity • Specificity

1 Introduction

Cyclic nucleotides play a central role in signal transduction as they act as second messengers (Beavo and Brunton 2002). The extracellular signals of hormones, growth factors, or physical stimuli are forwarded through receptor molecules in the plasma membrane to the inner cell, the cytoplasm, and the nucleus. Thereby, cells can react on environmental changes and stimuli. Signal transduction regulates cell growth, mitosis, metabolism, and apoptosis by coordinating cell physiology, traffic, as well as gene expression. Throughout the research of the 20th century, cAMP and cGMP set the tone in the cyclic nucleotide field (Beavo and Brunton 2002). However, recent studies revealed that the noncanonical cyclic nucleotides cCMP and cUMP are present in intact cells and organs (Burhenne et al. 2011; Hartwig et al. 2014; Bähre et al. 2015; Seifert 2015). In contrast, cXMP could not be detected so far in investigated cells, while the cellular presence of cIMP is controversial and could be dependent on specific pathophysiological conditions (Chen et al. 2014; Seifert et al. 2015). Noncanonical cyclic nucleotides have been shown to affect signal transduction effectors (Desch et al. 2010; Wolter et al. 2011; Hammerschmidt et al. 2012; Wolter et al. 2015; Seifert et al. 2015). However, this new branch of cyclic nucleotide research has not yet identified unique cellular effectors. Therefore, we summarize and discuss here the effects of these noncanonical cyclic nucleotides on the main effectors of cyclic nucleotide signaling, the cAMP-dependent protein kinase, and the cGMP-dependent protein kinase.

2 cAMP Signaling

In 1958, Sutherland and Rall isolated cAMP from liver cells and they soon described that cAMP was formed dependent on hormones such as epinephrine (Rall and Sutherland 1958; Sutherland and Rall 1958; Sutherland and Rall 1960). Consequently, Sutherland was awarded the Nobel Prize “for his discoveries concerning the mechanisms of the action of hormones” in 1971 and thereby introduced the concept of second messengers. Sutherland’s discovery opened the

broad field of cyclic nucleotide research. Nowadays, cAMP signaling serves as a classical model for signal transduction. While G protein stimulates adenylate cyclase to produce cAMP from ATP, specific phosphodiesterases degrade cAMP and thus turn off the second messenger signal. As a second messenger, cAMP regulates different cellular effector proteins that in turn mediate cellular and physiological answers. To these effectors belong the exchange protein directly activated by cAMP (EPAC), cyclic nucleotide-gated channels (CNG) and hyperpolarization-activated cyclic nucleotide-gated channels (HCN). However, the main cAMP effector is the cAMP-dependent protein kinase (PKA).

3 cAMP-Dependent Protein Kinase

Ten years after the discovery of cAMP, PKA was purified for the first time (Walsh et al. 1968; Beavo and Brunton 2002). PKA consists of two independent polypeptide chains, a regulatory (R) subunit and a catalytic (C) subunit (Fig. 1). In its inactive state, PKA forms a heterotetrameric complex (R_2C_2) of a regulatory subunit dimer and two catalytic subunits, respectively (Taylor et al. 2004; Taylor et al. 2013). The regulatory subunits contain an N-terminal domain (dimerization and docking domain, DD domain) which mediates the dimerization of regulatory subunits and the interaction with A-kinase-anchoring proteins (AKAPs) that localize PKA in complex with other proteins of the cAMP signalosome (Poppinga et al. 2014). An autoinhibitory domain (AD) binds to the catalytic cleft of the C subunit and thereby inhibits the phosphorylation of substrates. Two tandem cyclic nucleotide-binding domains (CNBs) that render PKA cAMP-dependent are localized at the C-terminus of each regulatory subunit. Binding of four molecules cAMP, a single cAMP per each CNB, is essential for activation of the heterotetrameric PKA complex. Cyclic nucleotide binding to the regulatory subunits induces the dissociation of the active C subunits. In mammals, four isoforms of regulatory subunits encoded by different genes are known: RIα, RIβ, RIIα, and RIIβ (Lee et al. 1983; Jahnsen et al. 1986; Scott et al. 1987; Clegg et al. 1988). Interestingly, type I isoforms (RIα and RIβ) inhibit catalytic subunits as pseudosubstrates that cannot be phosphorylated, while type II subunits (RIIα and RIIβ) are substrate inhibitors (Shabb 2001).

4 cGMP Signaling

cGMP was first discovered in rat urine by Ashman and coworkers (Ashman et al. 1963). Influenced by the cAMP research field, scientists soon described that cGMP formation is hormone-dependent. With the discovery of cGMP-specific PDEs and guanylate cyclases, it became clear that this cyclic nucleotide acts as a second messenger, too (Beavo and Brunton 2002). In 1998, Furchgott, Ignarro, and Murad were awarded the Nobel Prize for “for their discoveries concerning nitric oxide as a signalling molecule in the cardiovascular system” involved in cGMP

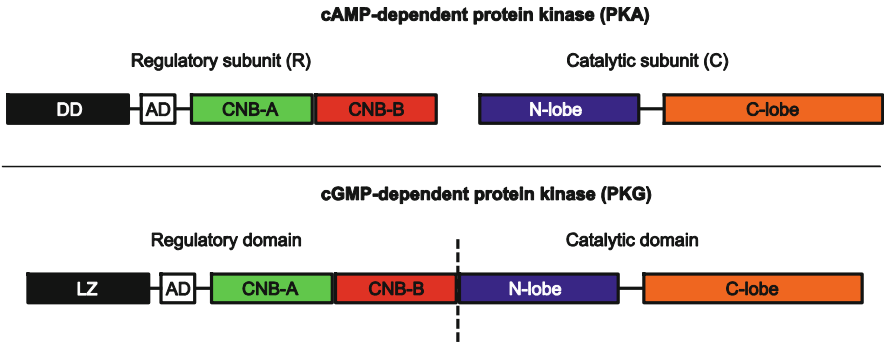

Fig. 1 PKA and PKG are homologous enzymes. Schematic domain organization of the cAMP-dependent protein kinase and cGMP-dependent protein kinase, respectively. PKA consists of a regulatory subunit (R) dimer and two catalytic subunits (C). The PKA R subunit has an N-terminal dimerization and docking domain (DD) followed by an autoinhibitory domain (AD). Two CNB domains (CNB-A and CNB-B) are localized at the C-terminus. In contrast to PKA, in PKG the regulatory and catalytic parts are localized as domains on one polypeptide chain. The border between the two domains is depicted as a dashed line. The N-terminal DD domain is replaced by a leucine zipper motif (LZ) again mediating dimerization

signaling. The classical cGMP signaling pathway can be described as follows: nitric oxide (NO) diffuses from endothelial cells into smooth muscle cells where it stimulates soluble guanylate cyclases to produce cGMP from GTP; cGMP then binds to and thus activates the cGMP-dependent protein kinase which in turn phosphorylates substrate proteins (Hofmann 2005); and, besides the main effector PKG, cGMP also regulates CNG and cGMP-regulated PDEs (Beavo et al. 1971; Kaupp and Seifert 2002).

5 cGMP-Dependent Protein Kinase

PKG was first described and purified from lobster tail muscle (Kuo and Greengard 1970). The domain organization of PKA and PKG shows a strong homology, but in contrast to PKA, the regulatory domain and the catalytic domain of PKG are fused on one polypeptide chain (Fig. 1). Additionally, PKG has an N-terminal leucine zipper motif mediating dimerization and interaction with proteins like G-kinase-anchoring proteins (GKAPs)(Corradini et al. 2015). In mammals, PKG is expressed from the two genes *prkg1* and *prkg2* (Kalderon and Rubin 1989; Wernet et al. 1989; Uhler 1993). The *prkg1* gene gives rise to two splice variants, PKG Iα and PKG Iβ, that differ in their first 80 to 100 amino acids, while *prkg2* only expresses PKG II.

Full-length PKG exists as a homodimer in which the catalytic domain is bound to the pseudosubstrate sequence of the autoinhibitory domain. Like in PKA, each PKG monomer has two tandem CNB domains and four molecules of cGMP are essential for full activation of the PKG dimer.

6 CNB Domains

CNB domains can be found throughout all kingdoms of life (Berman et al. 2005; Kannan et al. 2007; Mohanty et al. 2015). A conserved protein fold characterizes CNB domains (Fig. 2). Each CNB domain consists of helical and stranded parts: an N-terminal αA-helix is followed by an eight-stranded β barrel, which builds the binding pocket for the respective cyclic nucleotide. The domain ends with two helices, the αB- and αC-helix. The β barrel harbors the most conserved part of the CNB domain, the so-called phosphate-binding cassette (PBC). The PBC is localized between β sheets 6 and 7 and consists of 15 amino acids interacting with the sugar-phosphate moiety of the cyclic nucleotide bound (Diller et al. 2001; Canaves and Taylor 2002) (Fig. 2b, c). Therefore, the cyclic nucleotide kind of backs into its parking lot while the nucleobase faces the entrance of the binding pocket. In the PBC, a glycine residue forms a backbone interaction with the 2′-hydroxyl group of the ribose. In addition, a glutamate residue builds a hydrogen bond with this 2′-OH group. Additionally, a conserved arginine residue interacts with the equatorial oxygen of the cyclic phosphate. Since these residues are highly conserved, they do not contribute to cyclic nucleotide selectivity. Touching any of these residues drastically hampers the binding ability of the CNB domain (Øgreid et al. 1988; Bubis et al. 1988; Shuntoh and Steinberg 1991; Steinberg et al. 1991). Mechanistically, cyclic nucleotide binding to a CNB domain leads to conformational changes in the helical parts of the domain. In most cases, a so-called capping residue closes the binding pocket with the cyclic nucleotide bound. These residues are often aromatic and therefore interact with the nucleobase through π-stacking interactions.

The CNB domain of the catabolite activator protein from *E. coli* was the first of which a crystal structure was solved (McKay and Steitz 1981). Based on sequence alignments, homologous domains from PKA and PKG were modeled (Weber et al. 1987, 1989). These models revealed potential differences in substructures and in amino acid composition that could possibly mediate diverse binding mechanisms. Most of these models could be verified by the structures solved later on. The combination of cyclic nucleotide-free, so-called apo structures, with cyclic nucleotide bound structures allows the hypothetical description of binding-induced conformational changes.

7 Cyclic Nucleotide Specificity and Selectivity

In this chapter, cyclic nucleotide binding and cyclic nucleotide-dependent activation of PKA and PKG will be discussed in detail. We will focus on the cyclic nucleotide binding and cyclic nucleotide-dependent activation properties of PKA and PKG. Both kinases share two CNB domains of which the more N-terminal is designated as CNB-A, while the more C-terminal one is called CNB-B (Fig. 1). The rate of cAMP dissociation differs between these two domains by a factor of 5–10. Interestingly, the CNB-A in PKA is designated as “fast” and the CNB-B is

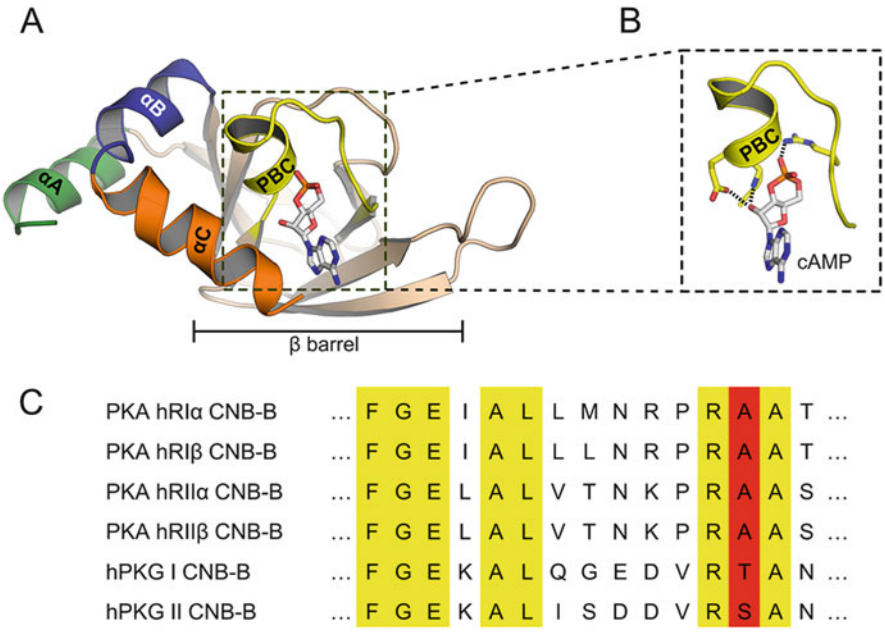

Fig. 2 The CNB domain. (**a**) Structure of the CNB-B of PKA hRIα with cAMP (PDB-No.: 1RGS; Su et al. 1995). The conserved structure of the CNB domain consists of α-helical parts (αA, αB, and αC) and a β barrel consisting of 8 β sheets. The cyclic nucleotide, here cAMP, is bound at the β barrel. The phosphate-binding cassette (PBC) interacts with the sugar-phosphate moiety of the cyclic nucleotide. (**b**) Zoomed view of the PBC with the cyclic nucleotide. Three conserved residues of the PBC form hydrogen bonds with the sugar phosphate of cAMP. (**c**) ClustalW2 sequence alignment of the PBC of CNB-B domains of PKA and PKG, respectively. Conserved sequences are shown in yellow. A key position mediating cAMP/cGMP selectivity is shown in red. Structure images were built using PyMOL (DeLano 2002)

designated as "slow cAMP exchange site." In PKG, the situation is reverse with the CNB-A being the "slow" and the CNB-B the "fast cGMP exchange site" (Rannels and Corbin 1980; Reed et al. 1996).

Although PKA and PKG are categorized by the respective cyclic nucleotide predominantly activating, each enzyme can also be stimulated by other cyclic nucleotides (Jiang et al. 1992; Von Bülow et al. 2007). However, significantly higher concentrations of cGMP in comparison to cAMP are needed to activate PKA, and vice versa PKG needs higher concentrations of cAMP than cGMP. A strong distinct specificity with a difference in affinity or potency of at least one order of magnitude defines selectivity. Only little is known about how this selectivity is achieved on the molecular level.

8 Molecular Mechanisms of Cyclic Nucleotide Selectivity

In the 1980s, selectivity profiles for activation potency were determined for PKA and PKG (Døskeland et al. 1983; Corbin et al. 1986). Furthermore, Corbin and coworkers described for PKG that the position 2 amino group of the guanine nucleobase is essential for high potency activation of PKG (Corbin et al. 1986). A key threonine/serine residue in PKG and cGMP-sensitive CNG CNB domains was predicted to form a specific hydrogen bond with the guanine 2-amino group (Weber et al. 1989). The cAMP-specific CNB domains of EPAC and PKA have an alanine residue at the corresponding position in the PBC, which cannot form a hydrogen bond with the amino group of the guanine base. Introduction of the threonine/serine residue into the CNB-B domain of PKA significantly decreased the cGMP activation constant compared to the wild type (Shabb et al. 1990). As the activation constant for cAMP remained unchanged, the mutated PKA lost selectivity but remained cAMP-specific. When the threonine residue was introduced into both CNB-A and CNB-B of PKA simultaneously, the cGMP activation constant was even lower (Shabb et al. 1991). With the cAMP activation constant slightly increased, the mutated PKA became unselective being activated by cAMP and cGMP with the same potency. Because cyclic nucleotide selectivity of PKA could not be switched, it can be assumed that residues other than the aforementioned threonine/serine in cGMP-sensitive proteins contribute to cGMP selectivity in PKG (Reed et al. 1996).

9 cGMP Specificity: cGMP-Selective Binding Domain

In the last few years, crystal structures of the PKG type I isoforms have been published (Osborne et al. 2011; Kim et al. 2011; Huang et al. 2014b). These structures reveal new insights into how cGMP is bound by the CNB domains and how discrimination between cGMP and cAMP is achieved on the molecular level. It was known for decades that PKG contains a high affinity and a low affinity binding site for cGMP. In PKG I, the CNB-A is the high affinity site, while CNB-B is the low affinity site (Mackenzie 1982; Reed et al. 1996; Kim et al. 2011; Huang et al. 2014b). However, the molecular mechanism of cyclic nucleotide selectivity remained enigmatic. Biochemical characterization of PKG runs far behind PKA research one reason because recombinant expression of mammalian PKGs in bacteria results in mostly insoluble and catalytically inactive protein (Feil et al. 1993; Aggarwal et al. 2011).

The crystallization of deletion constructs containing the CNB domains of PKG I along with functional studies identified that the CNB-A domain is unselective having only a twofold binding preference for cGMP compared to cAMP (Kim et al. 2011). The high nanomolar affinity toward both cGMP and cAMP was supported by the crystal structure of a regulatory domain fragment of PKG Iα (Osborne et al. 2011). This structure showed a cAMP molecule bound to the CNB-A, most likely due to a contamination during the recombinant expression in

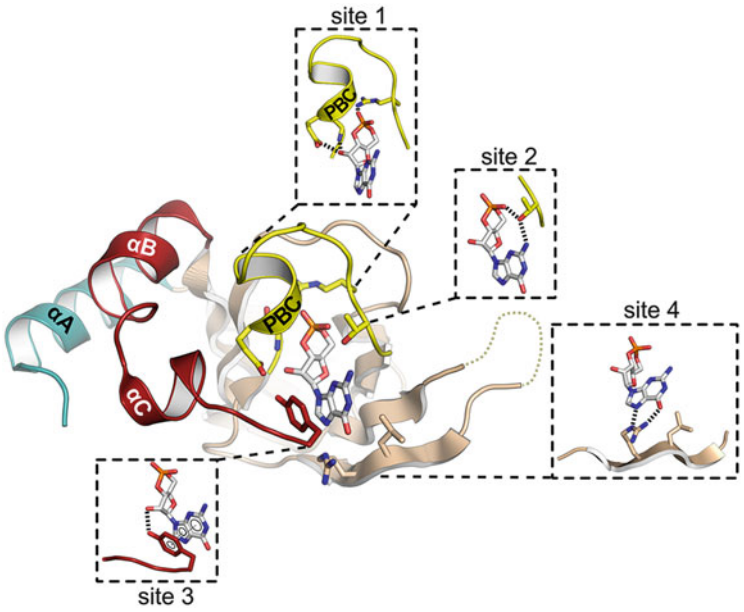

Fig. 3 A cGMP-selective CNB domain. Crystal structure of the CNB-B of PKG I with cGMP bound (PDB-No.: 4KU7; Huang et al. 2014b). Four sites of the binding pocket directly interact with the bound cGMP. Site 1 is the PBC, which forms hydrogen bonds with the sugar-phosphate moiety as described in Fig. 2. Site 2 is a threonine residue (T317) which hydrogen bonds the 2-amino group of the guanine nucleobase and the axial phosphate oxygen. Site 3 is a tyrosine (Y351) that forms a π–π stacking interaction with the nucleobase. Site 4 is localized at the beta sheet 5 where the side chains of two residues (L296 and R297) are pointing toward the guanine nucleobase. R297 forms hydrogen bonds with the 6-carbonyl oxygen and the position 7 nitrogen. Sites 2 and 4 contribute to cGMP specificity as the shown interactions cannot be formed with cAMP. Structure images were generated using PyMOL (DeLano 2002)

bacteria. A few years later, Huang and coworkers used biomolecular interaction analysis to identify the CNB-B of PKG I as highly selective for cGMP (Huang et al. 2014b). Along with the crystal structures published of the CNB-B with and without cGMP, four sites could be identified that directly interact with cGMP in the binding pocket (Fig. 3). Therefore, the guanine nucleobase is caught in between the clamp of the residues in beta sheet 5 and the threonine residue at the end of the PBC. The 2-amino group is lacking in the adenine nucleobase and the 6-carbonyl group is exchanged by an amino group. The different functional groups in positions 2 and 6 of the purine base are the basis for cAMP/cGMP selectivity. In line with this, mutation of the aforementioned leucine, arginine, and threonine residues to alanine in the CNB-B of PKG I dramatically reduced cGMP affinity, while cAMP affinity remained rather unaffected. Each single mutant led to a reduced selectivity for cGMP. The mutations also reduced the potency of cGMP to activate the full-length enzyme. In contrast, mutating the capping tyrosine residue to alanine reduced both cGMP and cAMP affinity and activation potency showing that this residue does not

mediate selectivity. Neutron diffraction of co-crystal structures of the PKG I CNB-B revealed that cGMP is bound in syn-conformation, while cAMP can be bound in both syn- and anti-conformation (Huang et al. 2014a, b). The same correlation was found for the PKG I CNB-A before (Kim et al. 2011). Thus, cAMP is not as fixed as cGMP in cGMP-specific binding pockets, which might be a reason for the reduced activation potency.

10 cAMP Specificity: cAMP-Selective Binding Domain

In contrast to cGMP, binding of cAMP is mainly based on hydrophobic interactions rather than hydrogen bonds as shown for the cGMP binding domains. The adenine ring is sandwiched from both sites by hydrophobic side chains (Su et al. 1995; Wu et al. 2004). The cAMP binding pockets are also closed by hydrophobic capping residues that form ring-stacking interactions with the nucleobases (Su et al. 1995; Wu et al. 2004). In PKA RIα, the capping residue in the CNB-B is a tyrosine just as in PKG I. Although hydrophobic interactions are significantly weaker than hydrogen bonds and salt bridges, the cAMP affinities of the PKA CNB domains are in the subnanomolar to nanomolar range (Hahnefeld et al. 2005; Moll et al. 2006a, b; Moll et al. 2007; Schweinsberg et al. 2008 and Table 1). The binding of cAMP can be described by hydrophobic interaction patterns where the hydrophobic nucleobase and the hydrophobic shell of the CNB domain congregate. However, thermodynamic analyses using isothermal titration calorimetry revealed that cAMP binding to the CNB-A and to the PKA RIα monomer is enthalpically driven indicating that the high affinity is mainly based on hydrogen bond formation (Moll et al. 2006b; Moll et al. 2007).

The affinities of the CNB domains in PKA RIα are switched in comparison to PKG I (Reed et al. 1996). The CNB-B is the high affinity site, while CNB-A has a lower affinity (Herberg et al. 1994).

Because crystal structures of PKA regulatory subunits with and without cAMP and in a holoenzyme complex with the catalytic subunit are available, individual steps of the cAMP-dependent activation mechanism have been predicted (Su et al. 1995; Wu et al. 2004; Kim et al. 2005; Hahnefeld et al. 2005; Kim et al. 2007; Bruystens et al. 2014). Furthermore, for the type I holoenzyme containing the RIα subunit biochemical data along with mutational analysis can be integrated into the activation model (Øgreid and Døskeland 1981a, b; Herberg et al. 1996; Boras et al. 2014). According to this model, the CNB-B of PKA RIα acts as a gatekeeper for activation. In the inactive holoenzyme, the CNB-A cannot be occupied because it is masked by the C subunit bound. Binding of cAMP to the accessible CNB-B induces conformational rearrangements that open the CNB-A. The CNB-A can switch between two conformational states, a C subunit bound state and a cAMP bound state, respectively. Thus, once cAMP is bound to the CNB-A, the C subunit is released and active to phosphorylate substrates.

The activation mechanism of other PKA holoenzyme complexes might be completely different as indicated by their individual quaternary structures

Table 1 Cyclic nucleotide binding to PKA RIα

	EC_{50} (nM)				
	cAMP	cIMP[a]	cGMP	cUMP	cXMP
Wild type	1	70	130	1,100	11,000
Δ1-91 (monomer)	2	70	250	900	24,000
CNB-A	42	n.d.	1,600	2,300	19,200
CNB-B	2	n.d.	1,100	3,900	8,900

Data were derived from surface plasmon resonance (SPR) interaction studies using solution competition experiments (Schweinsberg et al. 2008). PKA RIα constructs were preincubated with several dilutions of the respective cyclic nucleotide before residual binding to a high density 8-AHA-cAMP surface was monitored. The CNB-A construct consisted of amino acids 93-259 (Hahnefeld et al. 2005), and the CNB-B construct consisted of amino acids 252–359 (Shabb et al. 1995)

n.d. not determined

[a]The EC_{50} value for cIMP was derived from fluorescence polarization displacement assays using 8-Fluo-cAMP as transducer molecule as described in Moll et al. (2006a).

(Wu et al. 2007; Zhang et al. 2012; Ilouz et al. 2012). Comparable models for the PKG isoforms remain elusive as long as these enzymes cannot be crystallized with the catalytic domain present.

11 Specificity of Noncanonical Cyclic Nucleotides

Among the noncanonical cyclic nucleotides, cIMP and cXMP are most closely related to cAMP and cGMP. Both hypoxanthine and xanthine are purine bases like adenine and guanine. In the hypoxanthine nucleobase of cIMP, only the 2-amino group of guanine is missing (Fig. 4). Therefore, cIMP can be used to understand the role of this amino group for cGMP-specific binding. PKA RIα binds cIMP with a lower affinity than cAMP but with a higher affinity than cGMP (Table 1). Moreover, the regulatory domain and the CNB-B of PKG I bind cIMP with an affinity in between cGMP and cAMP (Fig. 5a, b). This emphasizes the importance of the 2-amino group of guanine for cGMP specificity. However, these results also show that other parts of the purine base additionally contribute to this specificity. Hypoxanthine and adenine differ in positions 1 and 6 (Fig. 4). The most obvious difference is that cIMP has a carbonyl group in position 6 just like cGMP, whereas cAMP has an amino group in this position. As mentioned above, the 6-carbonyl group interacts with arginine and leucine residues in β sheet 5 of the PKG I CNB-B, which mediates cGMP specificity (Fig. 3, site 4). Therefore, binding of cIMP to the CNB domains of PKG is most likely mediated by the conserved interactions of the sugar phosphate with the PBC and specific interactions between the position 6 carbonyl group and residues in β sheet 5 of CNB-B (Fig. 5c). Docking cIMP into the cGMP bound crystal structure of the PKG I CNB-B results in a model, in which cIMP is bound in syn-conformation and aligns with cGMP (Fig. 5c, d). In contrast to cGMP, the hydrogen bond between the 2-amino group and a threonine

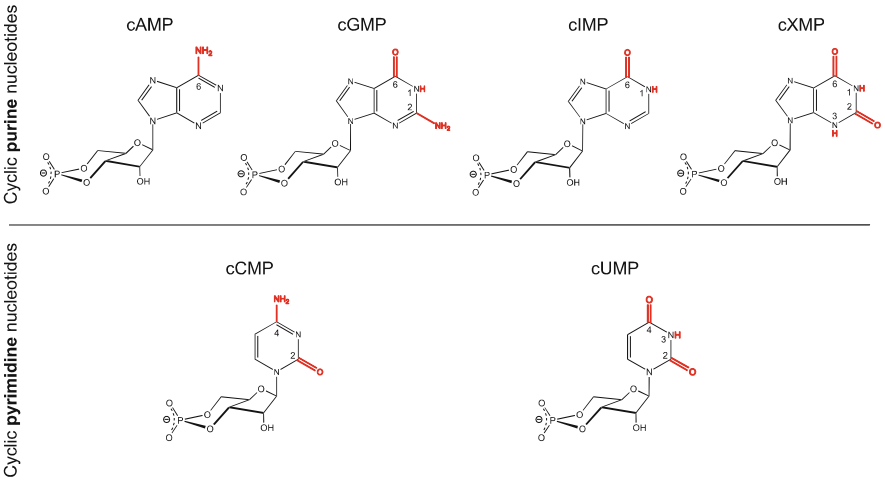

Fig. 4 Comparison of canonical and noncanonical cyclic nucleotides. All cyclic nucleotides have the same sugar-phosphate moiety. The nucleotides only differ by their nucleobase. The canonical cyclic nucleotides cAMP and cGMP belong to the cyclic purine nucleotides (*upper lane*) like cIMP and cXMP. cCMP and cUMP are cyclic pyrimidine nucleotides

residue is missing which contributes to the loss of affinity (Fig. 2c and Fig. 3, site 2). Interestingly, cIMP and cAMP have the same activation potency for PKG Iα indicating that the 2-amino group of cGMP is a stronger determinant for activation rather than binding (Table 3).

Along with this, cIMP has a tenfold higher potency than cGMP to activate the type I PKA holoenzyme (Table 2). Again, this underlines the importance of the 2-amino group of cGMP for cyclic nucleotide selectivity. Still, although this amino group is missing in cIMP, cAMP has a more than tenfold higher activation potency for PKA.

Although it is also a purine nucleotide, cXMP has the lowest affinity and activation potency for both PKA and PKG of all noncanonical cyclic nucleotides (Tables 1–3). Of all purines, the xanthine has the most modifications (Fig. 4). The combination of these moieties in positions 1, 2, 3, and 6 seems to have a negative effect on binding to CNB domains. Xanthine is more similar to guanine than to adenine as it also has a carbonyl group in position 6 and a hydrogen at the position 1 nitrogen. In contrast, xanthine lacks the 2-amino group critical for cGMP specificity and has a carbonyl group instead which might reduce affinity for PKG dramatically.

The two cyclic pyrimidine nucleotides cCMP and cUMP have a smaller nucleobase than the aforementioned cyclic purine nucleotides (Fig. 4). Therefore, the affinities for cCMP and cUMP should be relatively low. The pyrimidine nucleobases cannot fill the space of the CNB domains specific for purine nucleotides. The position and conformation of the nucleobases therefore should be less fixed and the possibility of a binding mode, which induces conformational

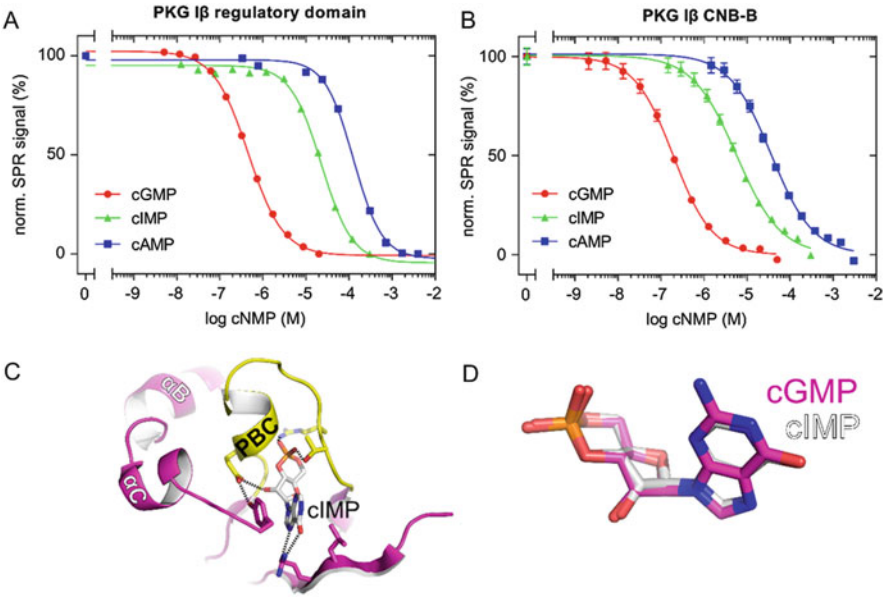

Fig. 5 cIMP has an intermediate affinity for PKG. Dose–response curves derived from SPR solution competition experiments on a high density 8-AHT-cGMP surface (Moll et al. 2006a). (**a**) The regulatory domain of PKG Iβ (amino acids 92-363) binds cGMP with nanomolar affinity, cAMP with a high micromolar affinity and cIMP with an intermediate affinity. (**b**) The isolated CNB-B of PKG Iβ (amino acids 219-369) is cGMP selective and binds cIMP with an affinity between cGMP and cAMP. (**c**) Binding pocket of the PKG Iβ CNB-B with a docked cIMP molecule. Docking was performed using AutoDock Vina (Trott and Olson 2010) with the cGMP bound structure of the PKG Iβ CNB-B (PDB-No.: 4KU7; Huang et al. 2014b). The docked cIMP represents the only docking model with a free energy ΔG of −10.5 kcal/mol. (**d**) Aligned cyclic nucleotides. The cIMP is docked in the same conformation like the co-crystallized cGMP molecule. Structure images were generated using PyMOL (DeLano 2002)

Table 2 Activation constants for the PKA RIα holoenzyme

	cAMP	cIMP	cGMP	cCMP	cUMP	cXMP
K_a (μM)	0.07	1	12	39	60	389
K_a'	1	0.056	0.006	0.002	0.001	<0.001

Data were modified from Wolter et al. (2011). Mean pEC_{50} values were transformed into K_a values. K'_a is the ratio of K_a(cAMP)/K_a(cNMP).

Table 3 Activation constants for PKG Iα. Data were modified from Wolter et al. (2011). Mean pEC_{50} values were transformed into K_a values. K_a' is the ratio of K_a(cGMP)/K_a(cNMP)

	cGMP	cIMP	cAMP	cCMP	cUMP	cXMP
K_a (μM)	0.15	19	15	13	71	105
K_a'	1	0.005	0.007	0.008	0.001	0.001

changes essential for enzyme activation, should be reduced (Seifert et al. 2015). PKA RIα binds cUMP with micromolar affinity. Interestingly, the monomeric and the full-length, wild-type PKA RIα bind cUMP with a significantly lower affinity compared to cGMP. The single CNB domains of PKA RIα bind both nucleotides with comparable affinities. Therefore, the coupling of two or even four CNB domains in the full-length protein allows the differentiation of cUMP and cGMP, resulting in a tenfold preference for cGMP. The reason for this might be the strong cooperativity of CNB-A and CNB-B in PKA RIα. CNB-A is only accessible when CNB-B is occupied. cGMP apparently has a higher potency to bind to CNB-B in a binding mode which allows to open CNB-A for cyclic nucleotide binding.

Both cCMP and cUMP are able to activate the PKA RIα holoenzyme (Table 2). The K_a values for both cyclic nucleotides are in the high micromolar range. In line with the relatively low affinity of cUMP, the activation potency of cUMP and cCMP is even lower than the potency of cGMP. Still, both pyrimidine nucleotides have a higher activation potency than the purine nucleotide cXMP. The activation potency of cCMP is twofold higher than the activation potency of cUMP.

Like PKA, PKG can be activated by cyclic pyrimidine nucleotides (Table 3). Again, cCMP and cUMP have high micromolar activation constants for PKG Iα which is in the same range as the K_a of cAMP. Therefore, PKG Iα has a strong preference for cGMP. All other cyclic nucleotides have a more than 100-fold lower activation potency than cGMP. PKG Iα cannot discriminate between cIMP, cAMP, and cCMP. Only cUMP has a significantly lower activation potency and cXMP has an even lower potency.

Besides the identification of cCMP as an agonist of PKA and PKG in vitro, the effects of synthetic cCMP analogs has been studied. The cell-permeant analog dibutyryl-cCMP (DB-cCMP) mediated vascular smooth muscle relaxation and platelet inhibition via PKG Iα in mouse intact cells (Desch et al. 2010). However, DB-cCMP failed to activate PKA in these cells. The major cellular metabolite of DB-cCMP is N^4-monobutyryl-cCMP (4-MB-cCMP). Interestingly, 4-MB-cCMP activates PKA RIα and RIIα more potently and more effective than PKG Iα in vitro (Wolter et al. 2014). Different cellular compartmentation between the PKA and the PKG signalosome, respectively, along with different accessibilities and membrane permeability for cyclic nucleotide analogs could explain the discrepancy between the in vivo and the in vitro results. In a pull-down approach using cCMP-coupled agarose, PKA R subunits and PKG isoforms were identified as cCMP-binding proteins (Hammerschmidt et al. 2012; Wolfertstetter et al. 2015). In addition, both PKA and PKG were stimulated by cCMP in tissue lysates. Further results indicate that cCMP-activated PKG regulates MAPK signaling. Therefore, the existence of a cCMP-dependent protein kinase was proposed (Wolfertstetter et al. 2015).

12 Conclusions

PKA and PKG are the main effectors of cyclic nucleotide signaling. Therefore, both kinases need to be tightly regulated. Binding of cAMP or cGMP to CNB domains releases the kinase domain from autoinhibition and thus enables phosphorylation of substrates. A and G kinases have a binding and activation specificity for cAMP or cGMP, respectively. The molecular basis for this specificity are key residues in the CNB domains. However, larger substructures and subunit crosstalk rather than single residues contribute to cyclic nucleotide selectivity.

Recently identified noncanonical cyclic nucleotides have been shown to act as agonists toward PKA and PKG. However, they display lower affinities and activation potencies indicating that in vivo kinase activation by noncanonical cyclic nucleotides relies on higher local concentrations. In a recent study, the basal concentrations of cCMP and cUMP in different cell lines were shown to be in the same range as the cGMP concentration, while the cAMP concentration was two to three times higher (Hartwig et al. 2014). With the physiological stimuli of cCMP, cIMP and cUMP production still unknown, the effective concentration of noncanonical cyclic nucleotides in a stimulated cell remains elusive. Therefore, we can only speculate about the physiological relevance of PKA and PKG activation by these noncanonical cyclic nucleotides in vivo. Interestingly, in a recent study, it could be shown that cCMP induces apoptosis in PKA, PKG, and HCN lacking cell lines (Wolter et al. 2015). This points out that unique effectors of noncanonical cyclic nucleotides still need to be identified.

Today's knowledge about the structure-function relationships of CNB domains can be used to improve affinities for noncanonical cyclic nucleotides through rational mutagenesis. In this way, specific sensors for noncanonical cyclic nucleotides could be designed.

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Regulation of HCN Ion Channels by Non-canonical Cyclic Nucleotides

Bryan VanSchouwen and Giuseppe Melacini

Contents

Abstract

The hyperpolarization-activated cyclic-nucleotide-modulated (HCN) proteins are cAMP-regulated ion channels that play a key role in nerve impulse transmission and heart rate modulation in neuronal and cardiac cells, respectively. Although they are regulated primarily by cAMP, other cyclic nucleotides such as cGMP, cCMP, and cUMP serve as partial agonists for the HCN2 and HCN4 isoforms. By competing with cAMP for binding, these non-canonical ligands alter ion channel gating, and in turn, modulate the cAMP-dependent activation profiles. The partial activation of non-canonical cyclic nucleotides can be rationalized by either a partial reversal of a two-state inactive/active conformational equilibrium, or by sampling of a third conformational state with partial activity. Furthermore, different mechanisms and degrees of activation have been

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observed upon binding of non-canonical cyclic nucleotides to HCN2 versus HCN4, suggesting that these ligands control HCN ion channels in an isoform-specific manner. While more work remains to be done to achieve a complete understanding of ion channel modulation by non-canonical cyclic nucleotides, it is already clear that such knowledge will ultimately prove invaluable in achieving a more complete understanding of ion channel signaling in vivo, as well as in the development of therapeutics designed to selectively modulate ion channel gating.

Keywords
Allostery • Binding domain • cAMP • Canonical • cCMP • cGMP • Conformational equilibrium • cUMP • Cyclic nucleotide • HCN • HCN2 • HCN4 • Ion channel • Isoform selectivity • Modulation • Non-canonical • Partial activation

Abbreviations

cAMP	Cyclic adenosine monophosphate
CBD	cAMP-binding domain of HCN
cCMP	Cyclic cytidine monophosphate
cGMP	Cyclic guanosine monophosphate
CHESPA	Chemical shift projection analysis
cIMP	Cyclic inosine monophosphate
cUMP	Cyclic uridine monophosphate
DEER	Double electron–electron resonance
GPCR	G-protein-coupled receptor
HCN	Hyperpolarization-activated cyclic-nucleotide-modulated ion channel
HSQC	Heteronuclear single-quantum coherence
IR	C-terminal intracellular region of HCN
MD	Molecular dynamics
NMR	Nuclear magnetic resonance
PKG	Protein kinase G
TD	Tetramerization domain of HCN
TM	N-terminal trans-membrane region of HCN

1 HCN Ion Channels: Introduction

The hyperpolarization-activated cyclic-nucleotide-modulated (HCN) proteins are cAMP-regulated ion channels that play a key role in nerve impulse transmission and heart rate modulation in neuronal and cardiac cells, respectively (Biel et al. 2002, 2009; Craven and Zagotta 2006; Zagotta et al. 2003; Kusch et al. 2010; Xu et al. 2010; Wang et al. 2002; Bois et al. 2011; Rosenbaum and Gordon 2004;

Johnson and Zagotta 2005). The HCN channels exist in four known isoforms, HCN1–4, of which HCN2 and HCN4 are the isoforms most significantly regulated by cAMP (Biel et al. 2002, 2009; Craven and Zagotta 2006; Xu et al. 2010; Bois et al. 2011; Johnson and Zagotta 2005). All HCN isoforms contain an N-terminal trans-membrane region (TM), which forms a tetrameric assembly harboring the ion pore, and a C-terminal intracellular region (IR) that confers regulation by cyclic nucleotides (Fig. 1a) (Biel et al. 2002, 2009; Craven and Zagotta 2006; Xu et al. 2010; Bois et al. 2011; Johnson and Zagotta 2005). It has been suggested that tetramerization of the HCN IR in response to cAMP binding is closely linked to up-regulation of ion channel opening (Zagotta et al. 2003; Craven and Zagotta 2004; Chow et al. 2012; Lolicato et al. 2011). The HCN IR tetramer observed in the presence of cAMP adopts an "elbow-shoulder" topology (Zagotta et al. 2003) involving four contiguous α-helices (αA′–αD′) at the IR N-terminus, which are referred to as the tetramerization domain (TD; Fig. 1a, b). A cAMP-binding domain (CBD) C-terminal to the TD allosterically controls self-association of the TD, whereby cAMP binding to the CBD promotes TD tetramerization (Fig. 1b, c) (Zagotta et al. 2003). However, it is currently not fully understood how cAMP promotes tetramerization, and in turn, ion channel up-regulation.

In a recent study by Akimoto et al. (2014a), the apo-state structure of the HCN4 CBD was solved by NMR, and was found to differ from the structure of the cAMP-bound CBD (Xu et al. 2010) by a rearrangement of the CBD α-helical subdomain similar to that observed for CBDs of other cAMP receptor proteins (Fig. 1d) (Huang et al. 2014; Kim et al. 2007; Su et al. 1995; Das et al. 2007; Das and Melacini 2007; Akimoto et al. 2013, 2014b; Abu-Abed et al. 2007; McNicholl et al. 2010). The study proposed a model of HCN4 allostery in which the apo-state CBD conformation destabilizes the tightly assembled tetramer through steric clashes with the TD (Akimoto et al. 2014a). These apo-state CBD/TD steric clashes are released through an increase in TD structural dynamics upon dissociation of the tetramer, thus favoring the dissociated TD in the absence of cAMP, and providing an explanation for the ion channel inhibition imposed by the apo-state CBD (Akimoto et al. 2014a). Similar structures were subsequently reported for the apo HCN2 CBD (Saponaro et al. 2014; Puljung et al. 2014). However, even after the structures of the apo and cAMP-bound CBD (Fig. 1b, d) were solved, only limited information was available on TD structural dynamics and on the pathways of inactive/active structural transition within the CBD (Akimoto et al. 2014a).

More recently, molecular dynamics (MD) simulations were utilized to further assess the influence of cAMP binding and tetramerization on the structural dynamics of the HCN4 IR and its domains (i.e., the TD and CBD), as well as the nature of the active/inactive conformational transition pathways explored by the CBD (VanSchouwen et al. 2015a). In particular, it was found that the TD becomes more flexible in the monomer states, thus removing steric clashes that the apo-state CBD structure would otherwise impose, in agreement with the hypotheses of Akimoto et al. (2014a). Furthermore, it was found that the apo CBD undergoes an active/inactive structural transition through a divergent manifold of pathways, while cAMP binding confines the CBD conformational ensemble to a tetramer-

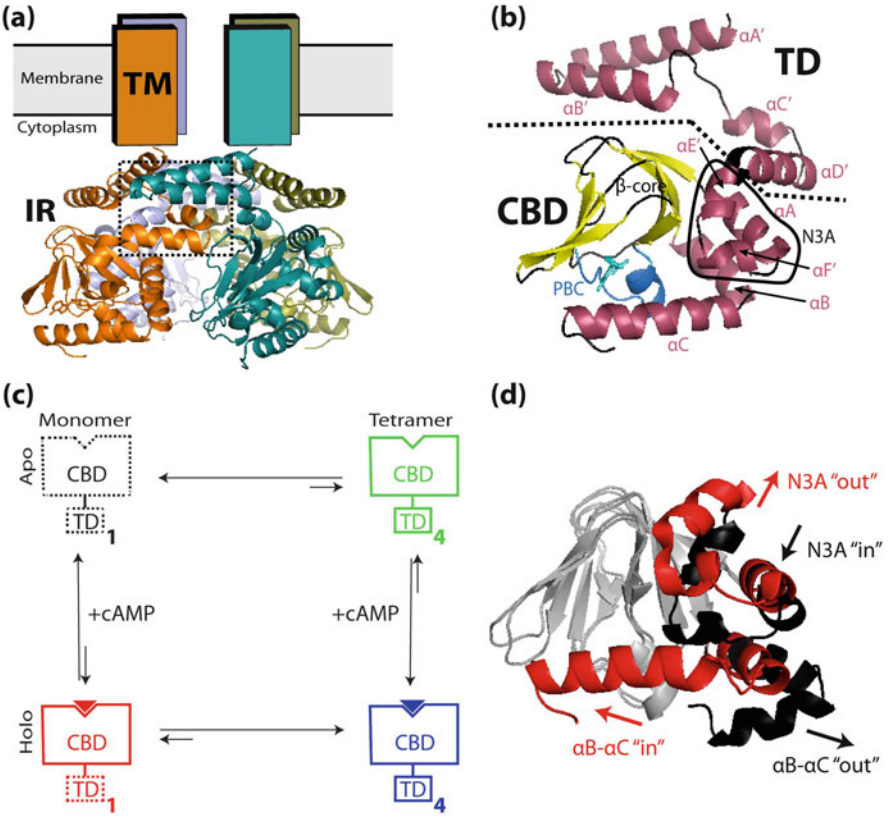

Fig. 1 (**a**, **b**) Overview of the HCN protein. (**a**) Tetrameric structure of the cAMP-bound HCN4 IR (RCSB Protein Data Bank code "3OTF") viewed parallel to the plane of the cell membrane. The four HCN4 monomers are shown in *orange*, *olive green*, *blue-gray*, and *teal*, and the tetramerization interface between the C-linkers of neighboring monomers is indicated (*dotted rectangle*). The four N-terminal trans-membrane (TM) regions, whose atomic-resolution structure is currently unknown, are shown as *rectangles*, while the four C-terminal intracellular regions (IR) are shown as *ribbon structures*. (**b**) Structural details of the HCN4 intracellular region for a single protomer (illustrated as a *ribbon structure*) with α-helices indicated in *maroon*, β-strands in *yellow*, and bound cAMP as *cyan sticks*. Boundaries between major structural regions are delineated by *dotted lines*, and key structural elements are indicated. The αA′–αD′ helices of the C-linker form the "tetramerization domain" (TD) of the IR, while the αA helix N-terminal to the β-subdomain, together with the αE′ and αF′ C-linker helices, forms the N-terminal "N3A" motif of the cAMP-binding domain (CBD). The phosphate-binding cassette (PBC), where cAMP binds, is indicated in *light blue*. (**c**) Outline of the thermodynamic cycle for the coupling between cAMP-binding and tetramerization of the HCN IR. The tetramerization domain (TD) and cAMP-binding domain (CBD) regions are schematically indicated as *rectangles*, and bound cAMP as a *solid triangle*. *Solid contour lines* indicate domain states with structures similar to the "3OTF" X-ray structure, while *dashed contour lines* indicate domain states with structures that are possibly different from the "3OTF" X-ray structure. The subscripts "1" and "4" refer to the number of protomers within each state. (**d**) Overlay of the cAMP-bound (*red ribbon*) and average apo (*black ribbon*) structures of the HCN4 CBD (RCSB Protein Data Bank codes "3OTF" and "2MNG," respectively; shown overlaid at their β-cores), illustrating the conformational changes that occur

compatible state, thereby priming the IR for tetramerization, and providing an explanation for how cAMP controls IR tetramerization (VanSchouwen et al. 2015a). Still, while these studies focused exclusively on cAMP, other cyclic nucleotides, such as cCMP and cUMP, are known to contribute to HCN channel gating either through direct binding or by competitively modulating the effect of cAMP.

2 HCN Ion Channels and Non-canonical Cyclic Nucleotides

The cCMP and cUMP cyclic nucleotides were previously found to be capable of promoting activation of the HCN2 and HCN4 isoforms, although maximal activation by such non-canonical ligands often occurs to only a partial extent and requires a higher ligand concentration compared to the canonical cAMP ligand (Akimoto et al. 2014a; Seifert 2015; Seifert et al. 2013, 2015; Zong et al. 2012; Beste and Seifert 2013; DeBerg et al. 2016). Still, the normal basal concentrations of cCMP and cUMP reside within a range sufficient for modulating HCN activity in cell types where HCN is present, including HEK293 astrocytes (which contain 32.6 ± 6.2 pmol cCMP/10^6 cells and 54.9 ± 11.3 pmol cUMP/10^6 cells) and sinoatrial pacemaker cells, with concentrations in some cases comparable to or approaching the respective basal concentrations of the known partial agonist cGMP (73.3 ± 5.2 pmol/10^6 cells in HEK293 astrocytes) (Seifert 2015; Zong et al. 2012; Seifert et al. 2015; Beste and Seifert 2013; Hartwig et al. 2014). Therefore, possible HCN modulation by cCMP and/or cUMP needs to be considered to achieve a more complete understanding of HCN function.

Often, non-canonical cyclic nucleotides function as partial agonists that compete with the respective canonical ligands for binding to CBDs, thus altering the canonical ligand-dependent activation profiles of the host proteins, and in turn modulating activity (VanSchouwen et al. 2015b). Indeed, similar concepts have been proposed for other eukaryotic cyclic nucleotide binding proteins. For example, it was previously suggested that intracellular signaling via protein kinase G (PKG) can be influenced through modulation of PKG activity not only by the canonical cGMP ligand, but also by the partial activator cAMP (VanSchouwen et al. 2015b; Francis and Corbin 1999; White et al. 2000). Furthermore, partial HCN activation by cCMP was previously proposed as a means of modulating heart frequency, while avoiding excessive stimulation of HCN by the canonical cAMP ligand (Seifert et al. 2015).

Fig. 1 (continued) within the CBD α-helical subdomain during cAMP-associated activation. Upon cAMP binding, the αB–αC region shifts from an "out" position to an "in" position, while the N3A shifts from an "in" position to an "out" position. For clarity, the β-cores of both structures are shown in *gray*, and the bound cAMP and αA′–αD′ helices from the "3OTF" structure are omitted. All *ribbon structures* were generated using Pymol (Schrödinger, LLC). Adapted from VanSchouwen et al. (2015a)

Another putative function of non-canonical cyclic nucleotides pertains to the tuning of isoform selectivity. For example, recent studies of cCMP binding to HCN4 (Akimoto et al. 2014a; Zong et al. 2012) versus HCN2 (DeBerg et al. 2016) have suggested that these isoforms may experience differing levels of activation (relative to cAMP) in response to cCMP binding (i.e., ~60% activation for HCN4 versus ~80% activation for HCN2). Therefore, the partial agonism of non-canonical cyclic nucleotides may provide a means for isoform-specific control of HCN channel gating.

3 HCN Partial Agonism by Non-canonical Cyclic Nucleotides: Putative Mechanisms

The partial activation of HCN observed in the presence of cCMP or cUMP can be explained by at least two main hypotheses. One postulates that the two-state inactive/active conformational equilibrium that rationalizes activation by the canonical ligand via conformational selection (Fig. 2a) is partially reversed in the presence of bound cCMP or cUMP. Thus, non-canonical cyclic nucleotides reduce

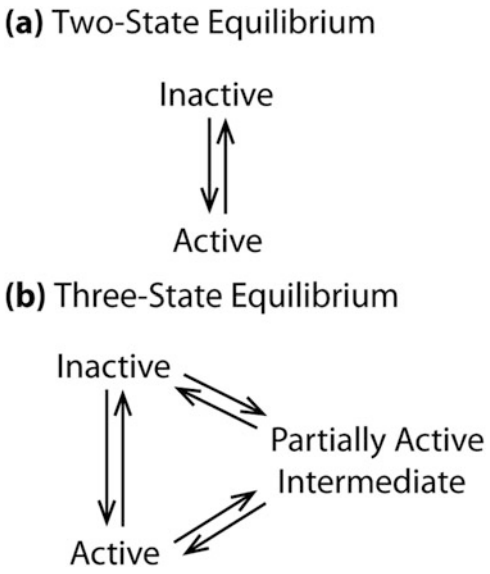

Fig. 2 Possible protein conformational equilibria hypothesized to explain partial activation by non-canonical cyclic nucleotides (VanSchouwen et al. 2015b). (**a**) Two-state inactive/active conformational equilibrium, which becomes partially reversed upon substitution of the canonical cyclic nucleotide ligand with a non-canonical ligand, thus reducing the proportion of the protein that resides in the active conformation. (**b**) Three-state conformational equilibrium, in which the non-canonical ligand-bound protein populates a third, partially active conformational state that is at least in part distinct from the canonical active and inactive conformations, thereby again reducing the proportion of the protein that resides in the active conformation

the overall level of activity by reducing the fraction of the protein that resides in the active conformation, whereby the extent of reduction observed under saturating conditions depends on the ratio of state-specific ligand association constants (VanSchouwen et al. 2015b; Moleschi et al. 2015). An alternative hypothesis posits that partial activation upon cCMP or cUMP binding is the result of the bound protein sampling a third conformational state that is at least partially distinct from both the canonical active and inactive conformations, and elicits only partial inhibition (Fig. 2b). Indeed, this latter explanation was previously found to rationalize the partial activation of PKG by cAMP, whereby population of a third conformation, and to a lesser degree the inactive conformation, in cAMP-bound PKG reduced the proportion of active protein, thereby reducing the overall level of PKG activity (VanSchouwen et al. 2015b). Likewise, recent ^{19}F-NMR and DEER analyses suggest that activation of G-protein-coupled receptors (GPCRs) proceeds through multiple intermediate conformational states, rather than a simple two-state conformational equilibrium (Manglik et al. 2015).

The former hypothesis is consistent with the data currently available for the partial agonism exhibited by cCMP with respect to HCN4 activation. Specifically, Akimoto et al. (2014a) identified linear patterns of chemical shift variations from apo to cCMP-bound to cAMP-bound HCN4, indicative of a two-state conformational exchange that is rapid in the NMR chemical shift time scale (Fig. 3a). Furthermore, the chemical shift variations suggested an average population of the active state at cCMP saturation that compares well with the extent of partial agonism independently measured through electrophysiology (i.e., ~60%; Fig. 3b, c). While a structural explanation of the partial activation by cCMP is currently not fully understood, binding site point mutations revealed that cCMP binds to the same binding site as cAMP (Zong et al. 2012). In addition, according to docking calculations (Zong et al. 2012), cCMP interacts with the same amino acid residues as cAMP upon binding, but the interactions of the base moiety with the binding site are altered somewhat, which may explain the lower potency and partial agonism of cCMP toward HCN.

In contrast to HCN4, the HCN2 isoform in the presence of cCMP exhibited signs of significant deviation from the two-state conformational equilibrium. Specifically, DeBerg et al. (2016) observed non-linear patterns of chemical shift variations from apo to cCMP-bound to cAMP-bound HCN2, indicating the presence of a conformational equilibrium consisting of more than two possible conformations. In addition, DEER analyses indicated that saturating amounts of cAMP, cCMP, and cGMP stabilized distinct conformational states within the CBD, confirming the presence of multiple possible conformations in equilibrium with one another (DeBerg et al. 2016). However, while DEER analysis revealed differing effects on the conformational ensemble of the CBD, free energies of activation computed from electrophysiology of integral HCN2 suggest that binding of these ligands leads to similar effects on ion channel opening (Table 1), pointing to the presence of a somewhat loose coupling between the cyclic-nucleotide-specific rearrangements of the CBD and the opening of the ion channel (DeBerg et al. 2016).

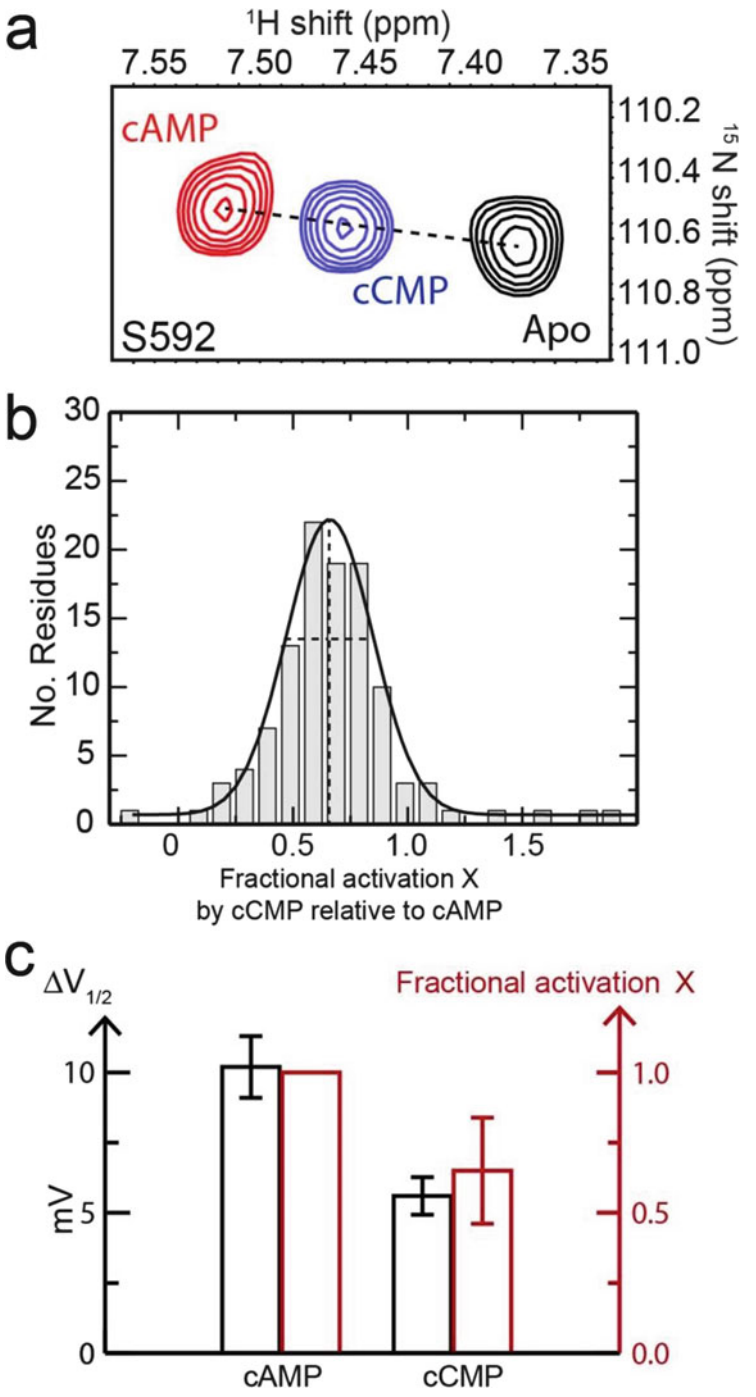

Fig. 3 Fractional activation by the partial agonist cCMP, as quantified by comparative analysis of apo, cAMP-bound, and cCMP-bound HCN4(563-724). (**a**) Representative HSQC cross-peak for HCN4(563-724) in the apo form, and bound to cAMP or cCMP. (**b**) The distribution of fractional

Table 1 Free energies of active-state stabilization for the intact HCN protein upon cyclic nucleotide binding, as estimated from DEER analysis of the isolated CBD, and from electrophysiology analysis of intact HCN[a]

Cyclic nucleotide ligand	ΔΔG of active-state stabilization from DEER	ΔΔG of active-state stabilization from electrophysiology
cAMP	$-12\ k_BT$	$-5.0\ k_BT$
cGMP	$-7\ k_BT$	$-4.4\ k_BT$
cCMP	$-10\ k_BT$	$-4.1\ k_BT$

[a]Values reported by DeBerg et al. (2016)

4 Concluding Remarks and Future Outlook

Although the HCN ion channels are regulated primarily by cAMP, other cyclic nucleotides such as cGMP, cCMP, and cUMP have been found to be capable of partially activating the HCN2 and HCN4 isoforms. By competing with the canonical cAMP ligand for binding to HCN2 and HCN4, these non-canonical cyclic nucleotide ligands may alter the cAMP-dependent activation profiles of these isoforms, and in turn, modulate their activity. The partial activation by these ligands could potentially be rationalized by either a partial reversal of the two-state inactive/active conformational equilibrium, or sampling of a third conformational state with only partial activity. Notably, the effects of cCMP binding were found to differ for HCN2 versus HCN4, and resulted in differing degrees of activation for these isoforms, suggesting that the partial agonism of the non-canonical cyclic nucleotides may provide a means for isoform-specific modulation of HCN.

Still, more work remains to be done in order to achieve a more complete understanding of HCN modulation by non-canonical cyclic nucleotides, and its relevance to HCN function. For instance, further investigation is needed to probe the mechanisms of how cyclic nucleotides such as cCMP exert differing effects on different HCN isoforms, and how different ligands are capable of exerting similar effects on the ion channel opening of HCN2 (Akimoto et al. 2014a; Zong et al. 2012; DeBerg et al. 2016). In addition, the mechanism by which cUMP influences HCN activity is not fully understood, and it is currently unknown whether other cyclic nucleotides such as cIMP influence HCN (Seifert et al. 2015). Finally, the influence of non-canonical cyclic nucleotides on other ion channels needs to be

Fig. 3 (continued) shifts toward activation for individual amino acid residues, as computed from chemical shift projection analysis (CHESPA) (Selvaratnam et al. 2012). The numbers of residues exhibiting a given fractional activation value are plotted, and the Gaussian fit to the distribution (*solid line*) is indicated. (**c**) Comparison between the average fractional activation observed for cCMP-bound HCN4(563-724), and the $\Delta V_{1/2}$ voltage shift caused by cCMP binding to full-length HCN4 channels as measured through electrophysiology (Zong et al. 2012). Adapted from Akimoto et al. (2014a)

investigated (Seifert et al. 2015). Understanding how non-canonical cyclic nucleotides contribute to the control of ion channel gating will prove invaluable in recapitulating the in vivo complexity of cyclic nucleotide signaling, as well as in the development of therapeutics designed to selectively modulate ion channel activity.

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Interaction of Epac with Non-canonical Cyclic Nucleotides

Holger Rehmann

Contents

Abstract

Epac1 and Epac2 are cyclic nucleotide-binding (CNB) domain containing proteins, which were originally identified as cAMP-regulated guanine nucleotide exchange factors (GEFs) for the small G-protein Rap. Therefore, Epac proteins founded next to protein kinase A (PKA) and cyclic nucleotide-regulated ion channels the third group of cAMP-responsive proteins in higher organisms. Epac proteins are involved in the regulation of several physiological processes. In particular Epac1 mediates the regulation of molecular processes underlying cell adhesion and mobility. In the pancreas activation of Epac2 potentiates the release of glucose-induced insulin secretion and received attention as a putative target for antidiabetic treatment. While the regulation of Epac by cAMP has been analysed in structural and biochemical detail, less is known on the interaction of Epac with non-canonical cyclic nucleotides. This chapter will discuss to what extent other cyclic purines than cAMP or cyclic pyrimidine could act as

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Epac agonists or antagonists. The focus will be on the biophysical analysis of the interaction between Epac and these cyclic nucleotides.

Keywords
Epac • Guanine nucleotide exchange factor • Rap • RapGEF • Small G-protein

1 Introduction

Small G-proteins cycle between a GDP- and a GTP-bound state. GEFs catalyse the release of the bound GDP which allows binding of GTP that is highly abundant under physiological conditions. Bound GTP is hydrolysed to GDP by the intrinsic GTPase activity of the G-protein. The catalytic efficiency of this process is accelerated by several orders of magnitude by GTPase-activating proteins (GAPs). Only the GTP-bound state is able to interact with effector proteins. This interactions results either in translocation of the effector protein or in its direct allosteric regulation. Since both the activities of GEFs and GAPs are subject to regulation, small G-proteins act in many signalling pathways as molecular switches, which integrate input signals (Vetter and Wittinghofer 2001; Bos et al. 2007).

Epac1 and Epac2 are GEFs for the small G-proteins Rap1A, Rap1B, Rap2A, Rap2B and Rap2C which are members of the Ras family (Kawasaki et al. 1998; de Rooij et al. 1998; Popovic et al. 2013). Both Epac proteins consist of an N-terminal regulatory region and a C-terminal catalytic region. The catalytic region contains a Ras exchange motif (REM) domain and a CDC25 homology domain (CDC25-HD), which sandwich a Ras association (RA) domain (Fig. 1a). The exchange activity is mediated by the CDC25-HD that interacts with Rap. REM domains always co-occur with CDC25-HD and stabilise the fold of the CDC25-HD by shielding hydrophobic residues (Quilliam et al. 2002). These residues are part of the scaffold that constitutes the catalytic site. RA domains interact with the GTP-bound form of small G-proteins from the Ras family (Herrmann 2003). Thus, the activation of Rap is coupled with the activity of other members of the Ras family. For example, active N-Ras recruits Epac2 to membrane compartments (Li et al. 2006). The regulatory region has an auto-inhibitory effect on the catalytic region, whereby the auto-inhibitions is relieved upon binding of cAMP. The regulatory region of Epac1 contains an N-terminal Dishevelled, Egl-10 and Pleckstrin (DEP) domain followed by a cyclic nucleotide-binding (CNB) domain (Fig. 1a). Epac2 contains an additional CNB domain N-terminal to the DEP domain. However, this CNB domain is neither required to maintain the auto-inhibited state nor is binding of cAMP to this domain required for activation (Rehmann et al. 2006). The basic regulation mechanism is thus the same in Epac1 and Epac2. In fact, a splice variant of Epac2 that lacks the N-terminal CNB domain exists.

While Epac1 is expressed rather ubiquitously, the expression of Epac2 is mainly limited to the pancreas and the central nervous system. Rap1 is known to promote

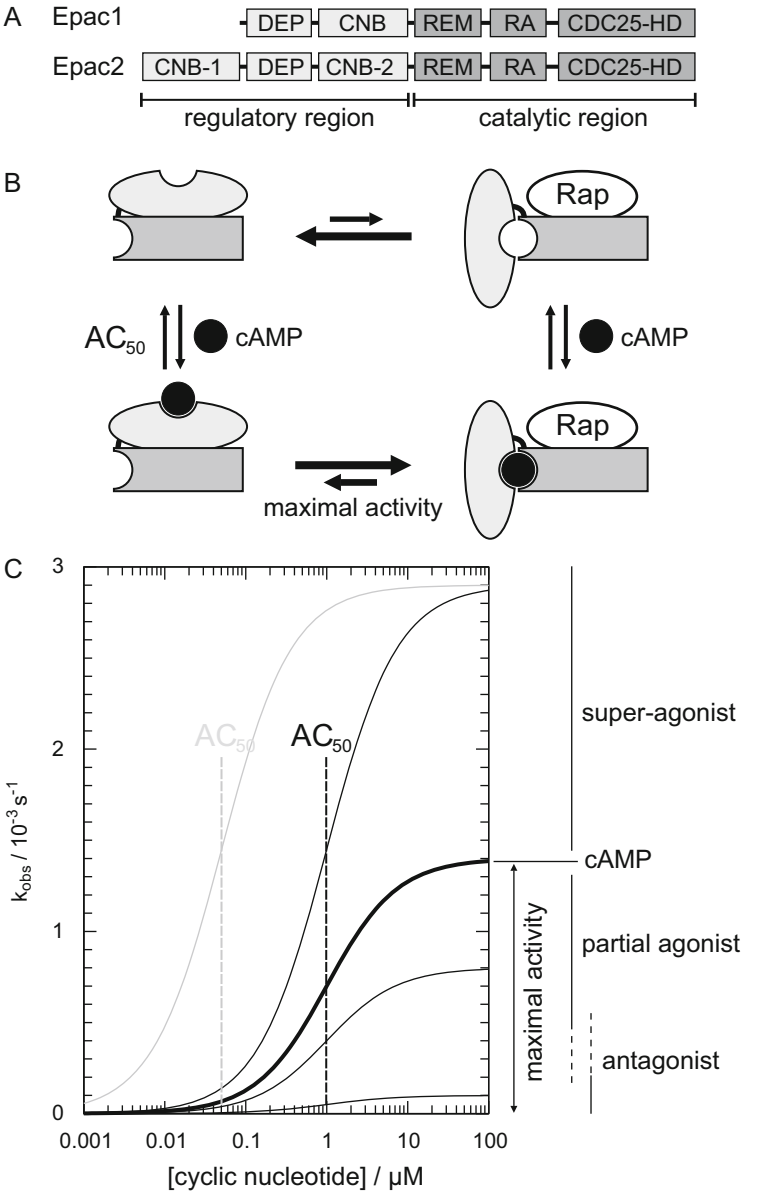

Fig. 1 Epac proteins as cyclic nucleotide receptors. (**a**) Domain organisation of Epac1 and Epac2. *DEP* Dishevelled, Egl-10 and Pleckstrin; *CNB* cyclic nucleotide binding; *REM* Ras exchange motif; *RA* Ras association; *CDC25-HD* CDC25 homology domain. (**b**) Conformational equilibrium of Epac. The regulatory region is depicted in *light grey* and the catalytic region in *dark grey*. The maximal activity, which is reached at saturating concentrations of a cyclic nucleotide, is a measure to what extent the cyclic nucleotide shifts the conformational equilibrium to the active side. (**c**) Dependency of the exchange activity on the concentration of cAMP (*bold line*) or various

cell–cell adhesion by regulating adherens junctions (Knox and Brown 2002), and it was shown that mainly Epac1 connects cAMP to Rap-induced reduction of endothelial barrier permeability (Wittchen et al. 2005; Cullere et al. 2005; Fukuhara et al. 2005). Epac1 is involved in mediating prostaglandin-induced endothelial barrier enhancement (Birukova et al. 2007) and can counteract the effect of platelet-activating factor (Adamson et al. 2008). To enhance the endothelial barrier, Epac1 impinges on vascular endothelial (VE)-cadherin (Cullere et al. 2005; Fukuhara et al. 2005; Kooistra et al. 2005; Noda et al. 2010) via inhibition of Rho signalling (Cullere et al. 2005; Adamson et al. 2008; Birukova et al. 2010). In cardiomyocytes Epac is involved in mediating β-adrenergic receptor-induced hypertrophy (Morel et al. 2005; Metrich et al. 2008) and increased contractility (Cazorla et al. 2009). To increase contractility Epac impinges via calmodulin kinase II and PLCε on intracellular Ca^{2+} concentrations (Cazorla et al. 2009; Oestreich et al. 2007, 2009; Pereira et al. 2007). By increasing the intracellular Ca^{2+} concentration in pre-Bötzinger neutrons, Epac stimulates bursting activity (Mironov et al. 2011; Mironov and Skorova 2011). In addition Epac2 influences the morphology of the central nervous system. Epac2 activity increases spine size (Woolfrey et al. 2009), promotes dendrite outgrowth (Srivastava et al. 2012) and is involved in axon guidance (Murray et al. 2009). Also the ability of Epac2 to potentiate glucose-induced insulin secretion was linked to its ability to increase intracellular Ca^{2+} concentrations (Kang et al. 2001, 2003; Idevall-Hagren et al. 2010), but in addition Epac was shown to increase the density of insulin granules at the plasma membrane (Shibasaki et al. 2007) and to promote the acidification of the granules (Eliasson et al. 2003).

2 Biophysical Basis for the Regulation of Epac

Crystal structures of Epac2 in the auto-inhibited state and of Epac2 in complex with cAMP and Rap were solved (Rehmann et al. 2006, 2008). The conformation of the catalytic region is the same in the inactive and the active state. However, in the inactive state, the CNB domain hangs over the CDC25-HD such that the access of Rap to the catalytic site is blocked. In the active state, the CNB domain is docked to the REM domain at the "back" of the CDC25-HD. Activation thus requires a movement of the CNB domain relative to the catalytic region. The core of the CNB domain moves as a rigid body, whereby the C-terminal α-helix of the CNB

Fig. 1 (continued) types of agonists and antagonists. Different affinities of the cyclic nucleotide towards Epac are reflected in changes in the AC_{50} (*grey line*, higher affinity than cAMP; *black lines*, same affinity as cAMP.) Cyclic nucleotides that induce a higher maximal activity than cAMP are classified as super-agonists, and cyclic nucleotides that induce a lower maximal activity than cAMP are classified as partial agonists or antagonists (see also Schwede et al. 2015; Rehmann 2006).

domain functions as a hinge. The helix melts partially to allow conformational rearrangements.

The activation process consists of multiple steps. First, cAMP approaches the core of the CNB domain, while the protein is still in the inactive state. This initial interaction is established between the phosphate-binding cassette (PBC), a short stretch of amino acid residues in the core of the CNB domain, and the phosphate–sugar moiety of cAMP. Some of the residues engaged in the interaction undergo a small conformational change to adapt an optimal docking pose. The conformation of the C-terminal hinge-helix is destabilised in consequence. This allows the core of the CNB domain to tumble driven by Brownian motion. If the core of the CNB domain has reached the docking position at the REM domain, interaction between the base of cAMP and a β-sheet is established. This β-sheet connects in primary as well as tertiary structure the CNB domain and the REM domain. The β-sheet could be considered as part of the CNB domain as well as of the REM domain. The interaction of the β-sheet with the base traps the core of the CNB domain at this position. The full cAMP binding site is thus only established after the core of the CNB domain has moved to the active conformation (Rehmann et al. 2007, 2008).

Studies with cAMP analogues support the model of the stepwise activation process. Competitive inhibitors are obtained by either substituting the equatorial oxygen of the phosphate by sulphur (Rp-cAMPS) or modifying the amino group at position 6 of the base (e.g. cPuMP and N^6-benzoyl-cAMP) (Rehmann et al. 2003). The sulphur substitution does not provoke the conformational change in the PBC, and thus the CNB domain remains at the inhibitory position. The amino group is involved in the interaction with the β-sheet. This interaction is abolished in cPuMP that lacks the amino group (Rehmann et al. 2008). Alternatively steric disturbance is obtained by coupling acyl or aryl groups to the amino group, as in N^6-benzyl-cAMP, N^6-benzoyl-cAMP or N^6-phenyl-cAMP (Rehmann et al. 2003; Schwede et al. 2015). These cAMP analogues interact normally with the PBC and allow the CNB domain to tumble, but since the CNB domain is not trapped at the docking position, it continues to hinder the interaction of Rap with the catalytic site.

As discussed, cAMP binding induces the transition from the inactive to the active conformation. The fundamental laws of thermodynamic postulate that conformational states are populated according to their energetic levels at equilibrium. Thermodynamically, cAMP binding shifts an equilibrium between an inactive and active conformation. This concept is illustrated in Fig. 1b. Indeed, even in the absence of cAMP, low exchange activity of Epac is detectable (Rehmann 2006). This activity is caused by a small fraction of Epac molecules in the active conformation in the absence of cAMP. Competitive inhibitors like the mentioned cAMP analogues are unable to efficiently shift the equilibrium to the active conformation. Thus, their binding results in a cyclic nucleotide-bound but inactive conformation. Interestingly, even upon binding of cAMP, a major fraction of Epac remains in an inactive conformation. Several cAMP analogues, like 8-pCPT-2′-O-Me-cAMP for Epac1 and Sp-8-BnT-cAMPS for Epac2, are able to shift the conformational equilibrium more efficiently than cAMP. Based on studies with cAMP analogues, it was estimated that only about 30% of cAMP-bound Epac1 and about 10% of

cAMP-bound Epac2 adapt the active conformation (Rehmann et al. 2003; Schwede et al. 2015; Rehmann 2006). This observation is of pharmacological interest as it demonstrates that the development of potent Epac agonist should be possible.

3 Interaction of Epac and Non-canonical Cyclic Nucleotides

Since Epac was identified as a cAMP-responsive protein, Epac-related research is focused on the function of Epac in cAMP-mediated signalling. However, not all CNB domains are only cAMP or cGMP selective. It was early recognised that the conductance in cilia of olfactory receptors is increased by cCMP only 20 times less efficiently than by cAMP or cGMP (Nakamura and Gold 1987). The interaction of non-canonical cyclic nucleotides and CNB domains of cyclic nucleotide-regulated ion channels is discussed in chapter "Regulation of HCN Channels by cCMP and cUMP". Also, PKA and protein kinase G (PKG) can be activated by the binding of non-canonical cyclic nucleotides (see chapter "cAMP-Dependent Protein Kinase and cGMP-Dependent Protein Kinase as Cyclic Nucleotitde Effectors"). This raises the question to what extent non-canonical cyclic nucleotides have the potential to either activate or inhibit Epac.

Two parameters are of importance to describe the interaction between Epac and a cyclic nucleotide, the affinity of the interaction and the extent to which the cyclic nucleotide shifts the equilibrium to the active site (Fig. 1c). The cyclic nucleotide can be classified as an antagonist that keeps the equilibrium on the inactive site, as a partial agonist that shifts the equilibrium less efficient to the active site than cAMP or as a "super-agonist" that shifts the equilibrium more efficiently to the active site than cAMP. Cyclic nucleotides often display different behaviours for Epac1 and Epac2, for example, 8-pCPT-cAMP is a partial agonist for Epac1 and Epac2, 8-pCPT-2′-O-Me-cAMP is a partial agonist for Epac2 and a super-agonist for Epac1 and Sp-8BT-cAMPS is a partial agonist for Epac1 and a super-agonist for Epac2 (Schwede et al. 2015). The limit from which a partial agonist is referred to as an antagonist is arbitrary and might be defined based on the ability of a cyclic nucleotide to inhibit an Epac-mediated physiological process. In any case efficient inhibition or activation requires a sufficient high affinity of the cyclic nucleotide.

A relatively simple experimental set-up allows classifying a cyclic nucleotide into one of the aforementioned categories. The exchange activity of Epac towards Rap is measured at a high concentration of the cyclic nucleotide and compared to the exchange activity induced by cAMP under saturating conditions. If the cyclic nucleotide induces strong exchange activity, a titration can be performed to determine the affinity from the concentration dependency of the exchange activity. If the cyclic nucleotide induces no or low exchange activity, two putative reasons need to be considered. First, the cyclic nucleotide could act as an antagonist or a partial agonist. Second, the cyclic nucleotide could be an agonist that binds Epac only with a low affinity. These two options are distinguished by adding the cyclic nucleotide together with cAMP. An antagonist or partial agonist inhibits the cAMP-induced

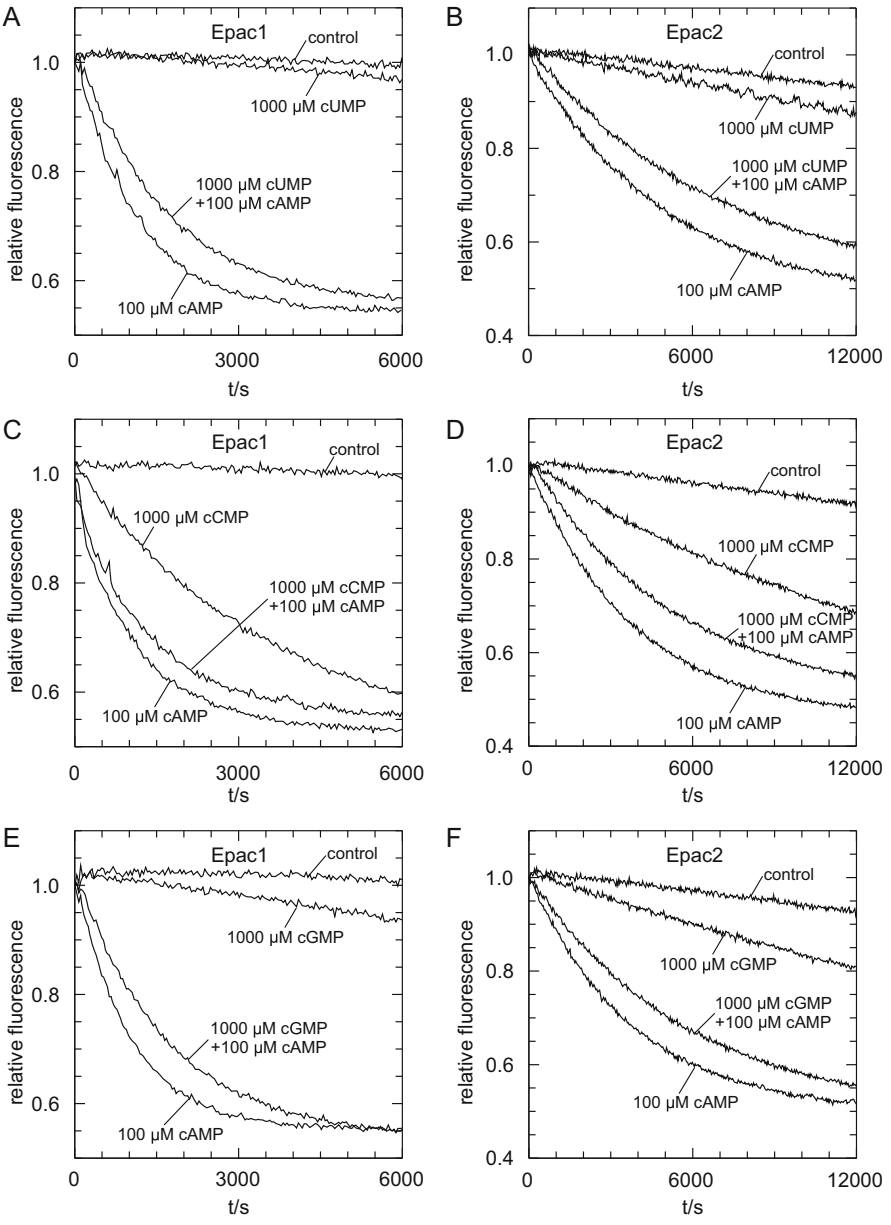

Fig. 2 Effect of cCMP, cUMP and cGMP on Epac-mediated GEF activity. The effect of cCMP (**a**, **b**), cUMP (**c**, **d**) and cGMP (**e**, **f**) on Epac1 (**a**, **c**, **e**)- or Epac2 (**b**, **d**, **f**)-mediated exchange activity in comparison to the effect of cAMP. A putative inhibitory effect was analysed by adding cCMP, cUMP or cGMP at tenfold higher concentration than cAMP to cAMP-activated Epac. To this end, the activity of recombinant Epac1 (aa 149-881, *Homo sapiens*) or Epac2 (aa 1-993, *Mus musculus*) towards Rap1B (aa 1-167, *Homo sapiens*) was analysed in a fluorescence-based assay (Rehmann 2006). Rap1B was loaded with the fluorescent GDP analogue 2′-/3′-O-(*N*-

exchange activity, whereas a cyclic nucleotide with low affinity leaves the cAMP-induced activity unaffected.

3.1 Epac and Cyclic Pyrimidines

cUMP-induced exchange activity of Epac1 and Epac2 is minor even if applied at 1000 μM (Fig. 2a,b). This suggests that cUMP is either an antagonist of Epac or is unable to bind to Epac. Furthermore, the exchange activity induced by 100 μM cAMP is slightly reduced in the presences of 1,000 μM cUMP (Fig. 2a,b). Thus, since cUMP neither activates nor inhibits Epac efficiently, its affinity for Epac is low. Saturation is clearly not reached at 1,000 μM; higher concentrations are experimentally not feasible. However, the experimental data show that if bound to Epac, cUMP shifts the conformational equilibrium less efficiently to the active site than cAMP. Thus, cUMP could be referred to as a "virtual" partial agonist, whereby this term reflects the low affinity. Similar results are obtained with cTMP (data not shown).

cCMP is a more potent Epac agonist than cUMP or cTMP. cCMP at a concentration of 1,000 μM induces clear activation of Epac1 and Epac2 although to a lesser extent than cAMP (Fig. 2c,d). At 1,000 μM, cCMP inhibits cAMP-induced activation and induces exchange activity to a similar extent as cUMP. Thus, the affinity of cCMP for Epac is higher than that of cUMP and cTMP, and cCMP shifts the equilibrium more efficiently than cUMP and cTMP. On the other hand, cCMP still binds Epac with lower affinity than cAMP and shifts the equilibrium less efficiently than cCMP. From titration experiments, the AC_{50} of cCMP was estimated to be about 400 μM for Epac1 compared to 40 μM for cAMP and to induce about 20% of the activity of cAMP under saturating conditions (Beckert et al. 2014).

3.2 Epac and Cyclic Purines

cGMP is as cAMP a well-established second messenger. cGMP activates protein kinase G (PKG) and certain cyclic nucleotide-gated channels. However, cGMP was found to be only a poor Epac1 agonist (Rehmann et al. 2003), and, indeed, cGMP does not efficiently activate Epac1 or Epac2 (Fig. 2e,f). Likewise, its ability to compete with cAMP and inhibit cAMP-induced Epac activation is limited. It is therefore unlikely that cyclic purines cGMP influences Epac signalling by direct

Fig. 2 (continued) methylanthraniloyl)-guanosine diphosphate (mGDP). The fluorescence intensity of Rap-bound mGDP is approximately twice as intense as free mGDP, and thus nucleotide exchange can be observed as decay in fluorescence upon addition of an excess unlabelled GDP. Reactions were performed in buffer containing 50 mM TrisHCl pH7.5, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 5% glycerol and 20 μM GDP at 20°C, with 200 nM Rap1Bl·mGDP and 100 nM Epac in the presence or absence of cyclic nucleotides as indicated. The graphs show the fluorescence intensity over time

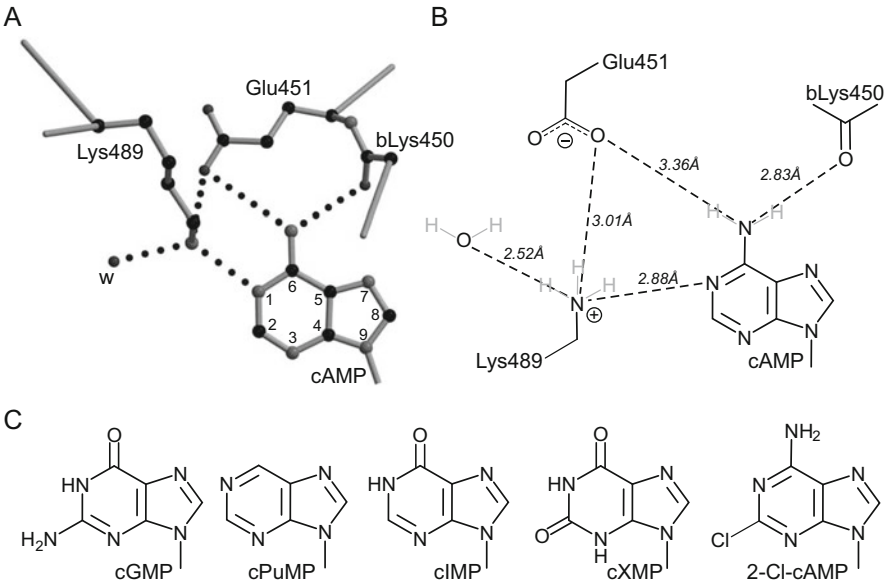

Fig. 3 Structural basis for the cAMP specificity of Epac. (**A**) Interactions between the adenine base and Epac. The base and interacting residues are shown in ball and stick representation. Hydrogen bonds are indicated by *dotted lines*. Amino acid residues are labelled by the *three-letter code*; *b* indicates that only the backbone is depicted. The atoms of the base are numbered; *w* water molecule. The figures are based on the protein database entry 3CF6 and were generated with the programmes Molscript (Kraulis 1991) and Raster3D (Merritt and Murphy 1994). (**B**) Schematic representation of the interaction. Hydrogen atoms are shown in *grey* and were placed assuming standard configuration. Hydrogen bonds are depicted as *dotted lines* between the non-hydrogen atoms and the distance is given in Å. (**C**) Chemical structure of purine bases

regulation, as cGMP, cIMP and cXMP have low affinity of Epac and are unable to shift the conformational equilibrium efficiently (Rehmann et al. 2003).

The amino group at position 6 of the purine base is a core requirement for Epac activation. This is in agreement with structural studies of Epac (Fig. 3). The amino group is engaged in a hydrogen bond with the backbone oxygen of Lys450 (Fig. 3A, B). The hydrogen atoms of the sp2-hybridised nitrogen are in the plane of the base. One hydrogen is pointing to one of the lone electron pairs of the backbone oxygen of Lys450. The other hydrogen is pointing to one of the oxygen atoms of the carboxyl group of Glu451, even though the distance of this interaction is rather long (Fig. 3B). Lys450 and Glu451 are part of the β-sheet at the C-terminus of the CNB domain. The interactions are only established after the CNB domain has moved to the active position. The interactions are thus contributing to an efficient shift of the equilibrium to the active site. An additional hydrogen bond that requires the active conformation is formed between the lone electron pair of the nitrogen at position 1 in the purine ring and the amino group of Lys489 (Fig. 3A,B).

cPuMP differs from cAMP only by the amino group at position 6 (Fig. 3C). Thus, cPuMP cannot be engaged in the interactions with the backbone oxygen of Lys450 and the side chain of Glu451. In consequence cPuMP is not able to stabilise the active conformation of Epac and acts as an antagonist. The same argument holds true for cIMP, in which the amino group is "replaced" by a "ketone oxygen" (Fig. 3C). This oxygen can only function as an acceptor of hydrogen bonds, while the amino group of cAMP functions as donor twice. Note, both the backbone oxygen of Lys450 and the side chain of Glu451 are acceptors and require a donor as partner. In addition, the lone electron pair at nitrogen 1 in cAMP is replaced by a hydrogen in cIMP. Thus, the interaction with Lys489 is lost. In agreement with these considerations is cIMP not able to stabilise the active conformation of Epac and act antagonistically (Rehmann et al. 2003).

Besides the missing amino group at position 6 are the bases of cGMP and cXMP "substituted" at position 2 (Fig. 3C). The bases of cGMP and cXMP are thus sterically more demanding. This demand is likely tolerated by Epac, as 2-Cl-cAMP activates Epac to a similar extent as cAMP.

4 Conclusion

Of naturally occurring cyclic nucleotides, only cAMP acts as strong Epac agonist. This is in agreement with the classification of Epac as a cAMP receptor protein (Kawasaki et al. 1998; de Rooij et al. 1998). Other cyclic nucleotides suffer from their limited ability to shift the conformational equilibrium of Epac to the active side. They are, in principle, partial agonists or even antagonists. However, their affinity for Epac is at least 10 times lower than that of cAMP, and no effects of other cyclic nucleotides than cAMP on Epac have been described in literature so far. Structural studies have pointed to a crucial role of the amino group at position 6 of cAMP in the activation process. This group is not engaged in interactions in PKA. This explains why PKA is less sensitive to changes in the base of the cyclic nucleotide than Epac.

With these qualifications, cCMP has still the highest potential to impinge on Epac signalling. cCMP is a partial agonist of Epac, and under certain physiological settings, it might either act as an Epac agonist or antagonist. In any case, high concentrations of cCMP would be a requirement. It should be appreciated that cyclic nucleotide signalling is subject to strong spatial regulation (Scott 2006). Whether or not such a scenario is real remains to be shown experimentally. Interesting in this context are high cCMP concentrations, which are generated by ExoY in lungs upon infections with *Pseudomonas aeruginosa* (chapter "ExoY as a Soluble Nucleotidyl Cyclase Toxin"), in particular since Epac functions in signalling in lung fibroblasts (Huang et al. 2008).

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Identification of cCMP and cUMP Substrate Proteins and Cross Talk Between cNMPs

Jens Schlossmann and Stefanie Wolfertstetter

Contents

Abstract

cCMP and cUMP are pyrimidine cyclic nucleotides which are present in several types of cells. These molecules could exert diverse cellular functions and might act as second messengers. In the last years, diverse approaches were performed to analyze possible cellular substrates and signaling pathways of cCMP and cUMP. In this review these approaches are summarized, and probable cross talk of these signaling molecules is described. These analyses might lead to the (patho)physiological and pharmacological relevance of these noncanonical cyclic nucleotides.

Keywords

cCMP • Cross talk • cUMP • Cyclic nucleotides • Kinases • Substrates

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Abbreviations

cCMP	Cyclic cytidine monophosphate
cUMP	Cyclic uridine monophosphate
MS	Mass spectrometry
PDEs	Phosphodiesterases
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase

1 Introduction

The pyrimidine cyclic nucleotides cCMP and cUMP are potential candidates as second messenger molecules. However, the functional analysis of cCMP and cUMP was a long time hampered by difficulties in the unambiguous identification of these molecules in cells and tissues (Newton et al. 1984). Recently, the use of LC-MS/MS methods revealed the presence of high concentrations of cCMP and cUMP in several mammalian cell lines and tissues (Bähre et al. 2015; Hartwig et al. 2014) and probable functions which were discovered (Desch et al. 2010). cCMP/cUMP was detected in some mammalian species, such as human, rat, and mouse (Hartwig et al. 2014). As mentioned above, high cCMP/cUMP levels were found in human HEK293 cells, in COS fibroblast-like kidney cells, and also in neuroblastoma cells (Hartwig et al. 2014). Interestingly rat astrocytes, hepatocytes, and endothelial cells contain high levels of cCMP/cUMP. Still it remained unclear whether these novel cyclic nucleotides can act as second messenger or whether they, due to the extremely low concentrations in vivo, can play a decisive role in physiological processes. A second messenger is an intracellular mediator which further contributes an extracellular signal of a first messenger (e.g., a neurotransmitter) into the cytosol (Hardman et al. 1971). One criterion for such a messenger is the formation by an enzyme that is regulated by a first messenger. Both pyrimidine cyclic nucleotides are formed by soluble nucleotidyl cyclases (sAC, sGC); membrane-bound cyclases are not involved (Bähre et al. 2014; Beste et al. 2012; Hasan et al. 2014) (Figs. 1 and 2). Second messengers are degraded by defined enzymes; in the case of cCMP/cUMP, these are PDEs (Monzel et al. 2014; Reinecke et al. 2011). Further criteria are the interaction with effector proteins, the mimicry of cCMP/cUMP functions by membrane-permeable cNMP analogs, and bacterial toxins (Seifert 2015; Seifert et al. 2015). Bacterial toxins like CyaA and EF exhibit a high adenylyl cyclase activity but only produce a small amount of cCMP (Gottle et al. 2010). The formation of cUMP is induced by CyaA, but not EF. ExoY is able to form both purine cyclic nucleotides cAMP and cGMP as well as pyrimidine cyclic nucleotides cCMP and cUMP (Beckert et al. 2014b). Consequently, some of the criteria for a second messenger have already been fulfilled. Therefore, it will be a major breakthrough to identify the signaling pathways and

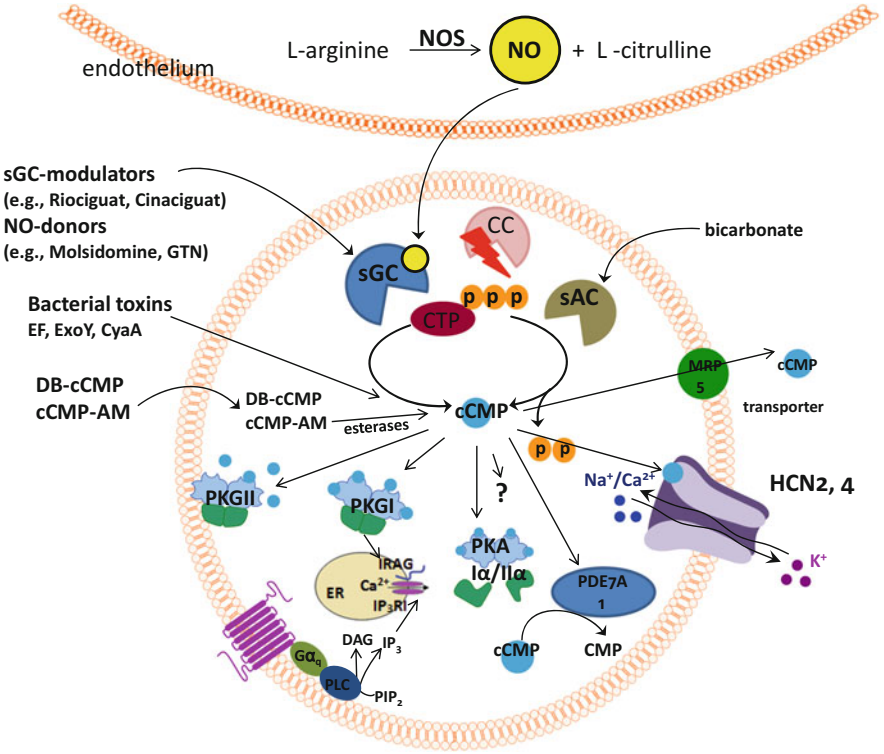


Fig. 1 Substrate signaling for cCMP. Schematic representation of a cell with possible attack points for cCMP and cross talk between the cNMP pathways. sGC and sAC generate cCMP from CTP. A specific cytidylyl cyclase (CC) does not exist according to current knowledge. cCMP engages at various targets in the cell and activates cAMP- and cGMP-dependent protein kinases (PKGI/PKGII or PKA) or opens voltage-gated cation channels (HCN2, HCN4). A cCMP-dependent protein kinase (cCK) has not yet been found. The cCMP degradation is proceeded via PDE7A1, the cCMP transport out of the cell via MRP5

function of these potential signaling molecules in cells and tissues. Meanwhile, several experimental setups were followed for the identification of cCMP and cUMP signaling substrates. These methods include the purification of cCMP-/cUMP-binding proteins and complexes by specific cyclic nucleotide affinity chromatography and by co-immunoprecipitation/ immunoprecipitation. Furthermore, the stimulation of protein kinase using specific kinase assays elucidates specific cNMP-mediated kinase substrates which can be further characterized by phospho-specific antibodies or radioactive phospho-incorporation. Several potential cCMP/cUMP substrates were identified with these assays (Figs. 1 and 2). These analyses also reveal cross talks between different cNMP signaling pathways. The review describes these approaches. Finally, an outlook will be given which will lead to future research directions in this exciting research field.

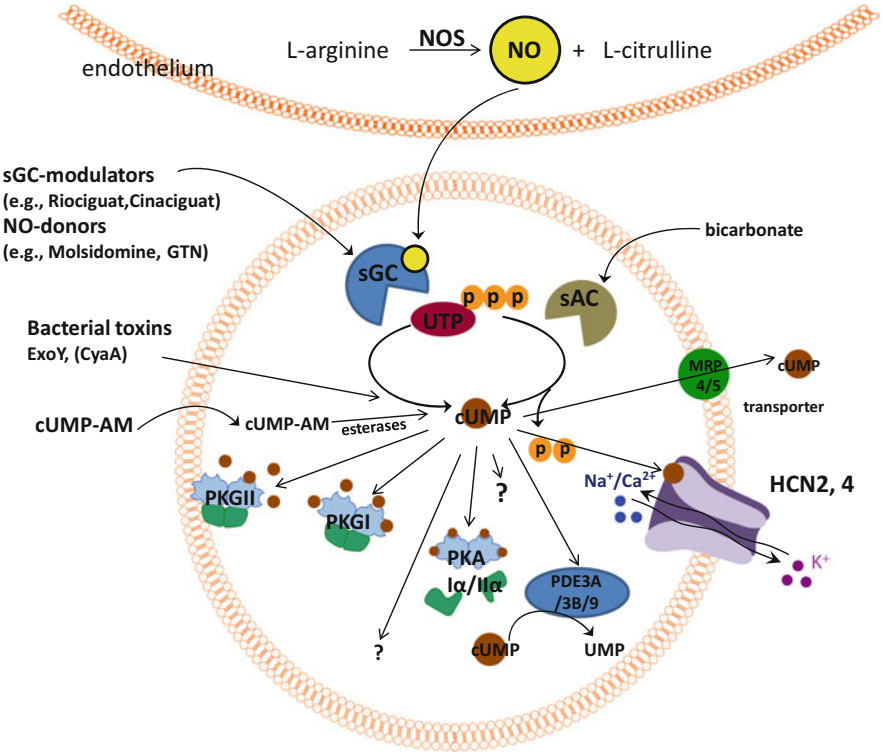


Fig. 2 Substrate signaling for cUMP. Schematic representation of a cell with possible attack points for cUMP and cross talk between the cNMP pathways. sGC and sAC generate cUMP from UTP. cUMP engages at various targets in the cell and activates cAMP- and cGMP-dependent protein kinases (PKGI/PKGII or PKA) or opens voltage-gated cation channels (HCN2, HCN4). The cUMP degradation is proceeded via PDE3A/PDE3B/PDE9, the cUMP transport out of the cell via MRP4/MRP5

2 Methods for the Analysis and Identification of cCMP/cUMP Signaling Pathways

The basis for the analysis and/or identification of cCMP/cUMP signaling pathways is the proper selection of the molecular source of the used material. Therefore, it is valuable to analyze cells and tissues where these pyrimidine cyclic nucleotides are found. However, the lack of pyrimidine cyclic nucleotides in the sourced material does not exclude binding or activation of proteins because the turnover of cyclic nucleotides is very high, and therefore these nucleotides could be not detectable. Recently, several cell lines (like HEK293 and B103 neuroblastoma cells) were tested which contain high concentrations of the cyclic nucleotides cCMP and/or cUMP (Hartwig et al. 2014). Therefore, it is conceivable that these cyclic nucleotides exert functional signaling pathways.

Diverse tools are presently used for the identification of cCMP-/cUMP-binding proteins. Binding assays are a good possibility to investigate protein-protein interactions and the affinity of a ligand toward the matrix. In particular affinity chromatographic methods are valuable for the analysis and identification of cCMP-/cUMP-binding proteins and protein complexes. This progress was possible because new cyclic nucleotide-coupled agarose materials were accessible for the use of affinity chromatographic procedures. In particular, purification of protein/protein complexes by cCMP-agarose affinity chromatography adapted from affinity methods using cGMP agarose (Koller et al. 2003; Schlossmann et al. 2000) or cAMP agarose (Hanke et al. 2011) was useful for the analysis and identification of substrate signaling pathways (Hammerschmidt et al. 2012; Wolfertstetter et al. 2015). Upon purification the bound proteins were identified by protein sequence analysis using LC-MS/MS mass spectrometry. In both approaches, the specific binding of proteins to the cCMP-coupled matrix was verified by lack of binding of the respective protein in the presence of excess cCMP. Meanwhile, some membrane-permeable cNMP analogs (dibutyryl-cNMP (DB-cNMP) or cNMP-acetoxymethylester (cNMP-AM)) are commercially available, which can mimic the effect of cNMP (Beckert et al. 2014a; Schwede et al. 2000). Since these analogs are highly lipophilic, they can easily get through the membrane into the cell interior, where they are cleaved by esterases.

2.1 Analysis

2.1.1 cCMP/cUMP Detection

Detection of cCMP/cUMP turned out to be tricky. Years ago, cCMP was identified using fast atom bombardment mass spectrometry (Newton et al. 1984). Later it was found that this method is too unsensitive and unspecific to detect the noncanonical cyclic nucleotides. Based on these setbacks, it was important to develop new highly sensitive and specific analysis methods for the cCMP/cUMP detection. In particular new mass spectrometry methods are a very suitable tool because of their low detection limits. Other ways to detect cCMP/cUMP are seldom reported. There is no specific antibody directed against cCMP/cUMP and therefore no enzyme-linked immunoassay kit available, as it exists for cAMP or cGMP. Furthermore, other well-proved methods for cAMP/cGMP quantification like HPLC or capillary electrophoresis are not sensitive enough for the detection of cCMP/cUMP. Furthermore the basal cCMP and cUMP concentrations vary extremely among the different systems. However, detection of pyrimidine cyclic nucleotides with HPLC coupled to MS/MS technology is possible (Beste et al. 2012). By coupling the two different techniques, many benefits arise. Advantage of this method is its high selectivity, specificity, and the fact that all analytes can be measured simultaneously (Oeckl and Ferger 2012). The process is automated and fast, and it is possible to measure both substance mixtures and purified compounds. In addition, there is no need for the use of radioactively labeled substances. So, the HPLC-MS/MS is an appropriate method for quantification and detection of cCMP/cUMP in in vitro and in vivo

investigations (Witters et al. 1997). After sample preparation and separation via high-pressure liquid chromatography, the mass spectrometry process is divided into further steps, including ionization (e.g., by electrospray ionization (ESI)), fragmentation, and mass detection of the analytes. In contrast, the HPLC-MS/TOF (time of flight) method is also used for cCMP/cUMP determination but is less sensitive compared to tandem mass spectrometry (Seifert et al. 2015). Interestingly working with isolated cell lines, the basal cCMP and cUMP concentrations are often comparable with the basal cGMP concentration though in isolated mammalian cells the cNMP levels are rather low (Bähre et al. 2015; Hartwig et al. 2014). However, the MS methods have limitations. Measuring tissue lysates and body fluids suppressed or missing signals may occur due to matrix effects. These effects are not observed using cell lysates and purified enzymes. For further information regarding cNMP quantification by HPLC-/LC-MS/MS, see Chap. 18 (Volkhard Kaever and Heike Bähre: Mass spectrometic details of cNMPs (3′5′ and 2′3′)). Another key technology for analysis of cCMP and cUMP functions is DMR (dynamic mass redistribution). It is a cell-based, label-free assay. Cellular changes result in relocalization of cellular matters, herein referred to as DMR which alters the optical density near the surface of a microtiter plate, which is detected by a biosensor. Using this technique Beckert et al. showed that cNMPs-AMs mimic bacterial nucleotidyl cyclase cytotoxicity (Beckert et al. 2014a). These analogs can pass the membrane and are prodrugs, in which the phosphate group is protected by an acetoxymethylester (Schultz et al. 1994). ExoY is a toxin from *Pseudomonas aeruginosa* and has adenylyl cyclase activity. It massively increases the cGMP and cUMP levels and also in a smaller manner cAMP and cCMP. The *Bordetella pertussis* toxin also increases cyclic nucleotides, like cAMP and cCMP (Beckert et al. 2014b). cCMP-AM was also used for analysis of mouse lymphoma cells indicating apoptotic properties of cCMP (Wolter et al. 2015).

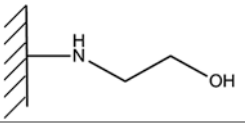
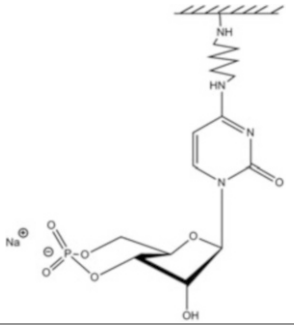
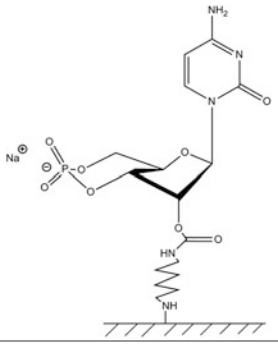
2.1.2 Interaction Proteins

To identify cNMP-binding proteins from tissue lysates, it is able to work with agaroses which are coupled to cyclic nucleotides (cNMPs) via linkers (ethanolamine) (Corrie et al. 1992; Hanke et al. 2010). It is possible to directly immobilize the ligands (e.g., cyclic nucleotides) on the affinity matrix. Here the ligands can capture their specific interaction partners and bind them mostly non-covalent. Usually the bound proteins are eluted using buffer or free ligands. Different linker lengths and different attachments of cNMP to the agarose affect the interaction of the proteins with cNMP (Hammerschmidt et al. 2012). The principle is similar to the affinity chromatography, with the exception that in addition always a competition, which is a displacement reaction, is carried out concomitantly to the cNMP precipitation. This method allows to concentrate low abundant proteins and to deconcentrate high abundant proteins (Aye et al. 2009). Proteins can bind nonspecifically or specifically to the cNMP-coupled matrix. Through a preincubation with a BSA solution, these nonspecific bindings can be minimized. The matrix is then added to the lysate to be tested. When an excess of cNMP is added, the specific bonds are solved and bind to the "free" cNMP. The specific binding proteins are now in the supernatant and can be removed. The subsequent elution with buffer thus

solves only nonspecifically bound proteins from the matrix. Ideally, the specifically bound proteins concentrate in the eluate. As a negative control (Ctrl.), ethanolamine-agarose was used. Nevertheless working with very large proteins, steric interactions can occur which can interrupt the ligand-protein interaction. Unfortunately very weak protein-ligand interactions are often removed during the washing steps and cannot be detected with this method. Moreover proteins with very high binding affinity cannot be solved from the matrix, even if a high amount of free ligand is used for elution. There are several commercially available cCMP agaroses. A list of these agaroses can be found in Table 1. cUMP agaroses are not yet commercially available. In 2′-2-aminoethylcarbamoyl-cCMP (2′-AHC-cCMP), the cCMP is linked to the matrix via the 2′-O-ribosyl group; in 4-6-aminohexyl-cCMP (4-AH-cCMP), it is the 4-NH group of the pyrimidine ring system (Table 1). In experiments with 2′-AHC-cCMP agarose, no or a weaker binding of proteins to the matrix was observed. The reason for this may be steric problems or the fact that the 2′-hydroxy group is essential for the interaction with proteins. Hammerschmidt et al. (Hammerschmidt et al. 2012) analyzed extract proteins from cellular sources with 2′-AHC-cCMP agarose, 4-AH-cCMP agarose, and the corresponding EtOH-NH control agarose. In several mammalian cell lines (HELA, HL-60, B103, HEK293), the authors described specific binding of cAMP-kinase regulatory subunits RIα and RIIα. Wolfertstetter et al. (Wolfertstetter et al. 2015) used 4-AH-cCMP agarose and EtOH-NH agarose for the analysis of tissue extracts (jejunum and lung). Besides PKA regulatory proteins, there were several further proteins which could be identified, namely, cGMP-dependent protein kinases I and II (PKGI and PKGII). The identification of PKGI was performed by mass spectrometry. PKGII binding was shown by specific antibodies directed against this protein. Furthermore MAPK was identified as a cCMP-interacting protein via immunoblot (Fig. 3). These results show that less abundant proteins are overlooked by the LC-MS/MS-based protein sequence analysis approach. Utilizing several distinct tools for the detection is the most precious way for cyclic nucleotide substrate identification.

In contrast to the technique described above, the CCMS (capture compound mass spectrometry) technology is carried out under mild conditions and is therefore suitable to detect low-affinity protein interactions. With this new method, proteomic studies are improved by reducing background proteins. Capture compounds are molecules which are build up of three different parts. The first part is called the selectivity function and it is able to interact with specific proteins. The second part is the reactivity function, which binds these proteins by a covalent bond and which is activated by UV light. Finally the sorting function or pullout function enables the isolation of the captured proteins from the mixture (Fischer et al. 2011; Koster et al. 2007). The characterization of the bound proteins is usually carried out by LC-MS/MS. cAMP and cyclic di-GMP capture compounds were successfully used for the identification of substrate proteins (Luo et al. 2009; Nesper et al. 2012). It is conceivable that cCMP or cUMP capture compounds are suitable for the characterization of interactors with these cyclic nucleotides.

Table 1 Used cCMP and control agaroses

Agarose	IUPAC nomenclature	Structure
EtOH-NH agarose	Ethanolamine-agarose (control agarose)	
4-AH-cCMP agarose	N^4-(6-Aminohexyl)cytidine-3′,5′-cyclic monophosphate agarose	
2′-AHC-cCMP agarose	2′-O-(6-Aminohexylcarbamoyl)cytidine-3′,5′-cyclic monophosphate agarose	

For detecting cNMP-dependent protein complexes and protein-protein interactions, one can apply the method of co-immunoprecipitation. Here advantage of an antigen-antibody reaction is taken. The antibody directed against a protein is coupled to a solid stationary phase and binds the antigen from a protein mixture or tissue lysate. In addition, all interaction partners of the antigen are precipitated, the so-called coprecipitates. As stationary-phase Sepharose beads are used, these are coated with protein A. Protein A is recovered from the cell wall of *Staphylococcus aureus* and binds well to the Fc portion of human IgG, but is also suitable for all rabbit antibodies. Immunoprecipitation can be performed under denaturing or non-denaturing conditions. In a precipitation under denaturing conditions, the proteins are first treated by SDS (0.5–1 %) and heat and then bound to the antibody-coupled Sepharose. Precipitation under non-denaturing conditions is carried out in the presence of non-denaturing detergents such as lubrol (nonaethyleneglycol monododecyl ether) or Tween-20 (polyoxyethylene sorbitan monolaurate) or completely carried out without detergent. As the binding is more specific, the more sodium chloride is included in the precipitation buffer.

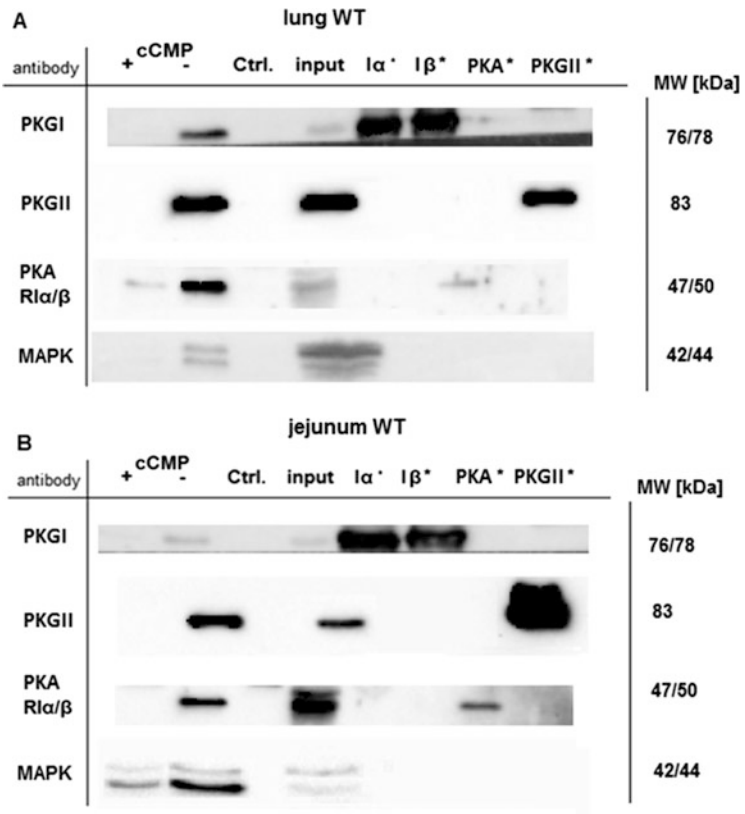


Fig. 3 Identification of cCMP-binding proteins in different WT tissue lysates. (**a**) Lung tissue lysates were incubated with 4-AH-cCMP agarose or EtOH-NH agarose (Ctrl.). For the competition, 200 μM cCMP was added ("+") or was replaced by water ("−"). The detection of the bound proteins was performed by SDS-PAGE and Western blotting, as control total lysate ("input," 1 μg/μl) and purified enzymes (1.5 ng/μl) were applied. The experiment was carried out similarly to (**a**) with (**b**) jejunum tissue lysate (figure according to Wolfertstetter et al. 2015)

After the unambiguous detection of cCMP and cUMP, it is obviously important to find more cNMP-binding proteins to elucidate a physiological function of the cyclic nucleotides. For this purpose, besides the cNMP agarose studies and other proteomic studies, also the so-called "cofactor fingerprinting," coupled with a STD-NMR (saturation transfer difference nuclear magnetic resonance spectroscopy) method, is used, whereby, inter alia, receptor-ligand binding processes can be elucidated (Dalvit et al. 2002; Mayer and Meyer 2001). Using this method, RSP2 (radial spoke protein-2) was identified as a cCMP-interacting protein (Yao and Sem 2005).

2.1.3 Substrate Proteins

Phosphorylation is a key step for the regulation of protein function. In order to demonstrate the stimulating effect of cyclic nucleotides on kinases, the method of phosphorylation can be carried out. Cyclic nucleotides, like cGMP or cCMP, are able to activate serine/threonine kinases which are so-called transferases (Pfeifer et al. 1999). They catalyze the transfer of (γ)-phosphate groups from ATP to hydroxyl residues of the substrate protein. To make this phosphate transfer visible, either general biochemical (Western blotting and phospho-specific antibodies) or isotope-based models are possible. A traditional technique to study phosphorylated proteins and to determine the catalytic activity of protein kinases is using ATP in which the terminal phosphate group is radioactively labeled (e.g., by ^{32}P). Phospho-specific antibodies are polyclonal or monoclonal antibodies that detect target proteins which are phosphorylated on a tyrosine, threonine, or serine residue. However, often it is difficult to generate really specific antibodies, and so during the last years label-free techniques to detect protein-protein interaction emerged. Phosphoprotein enrichment assays are a suitable and efficient tool for purification of phosphorylated proteins by affinity chromatography. Using commercially available kits (e.g., Qiagen), phosphorylated proteins can be separated from non-phosphorylated proteins from cell culture lysates or tissue extracts; thereby cell signaling can be examined. The kits usually contain columns, buffers, reagents, and ultrafiltration columns. A great advantage is that this technique is noninvasive and compatible with mass spectrometry, Western blotting, and two-dimensional gel electrophoresis. Furthermore phospho-proteomic approaches are a good clue for the detection of new drug targets (Fila and Honys 2012).

2.2 Identification

To identify and characterize bound proteins, the eluate (of the cCMP-agarose affinity chromatography) is separated via electrophoresis, and silver or Coomassie staining can be used to make the proteins visible. Silver staining is based on a method that was published in 1973 by Kerenyi and Gallyas (Kerenyi and Gallyas 1972). In comparison to Coomassie staining (detection limit 10 ng (Weiss et al. 2009)), the silver staining is more sensitive. The detection limit is less than 1 ng of protein per band (Weiss et al. 2009). However, it is also less reproducible and susceptible to interference. For further analysis, the protein bands that should be identified were excised from the gel and further processed. Finally, the peptides extracted from the gel were characterized by nano-LC-MS/MS (see above and in more detail Chap. 18 (Volkhard Kaever and Heike Bähre: Mass spectrometic details of cNMPs (3′5′ and 2′3′))).

3 Potential Substrates of cCMP/cUMP Signaling

The recent identification of cCMP in some cell lines and mammalian cells by a highly sensitive and specific LC-MS/MS method led to the search for cCMP-interacting proteins. According to current knowledge, there is no specific cytidylyl cyclase, converting CTP in cCMP. Though sGC and sAC show a cCMP-generating activity (Beste et al. 2012; Hasan et al. 2014), membrane-bound cyclases are not involved in cCMP formation (Hasan et al. 2014). cCMP activates the regulatory subunits RIα and RIIα of PKA (Wolter et al. 2011) and both PKG isoforms (Wolfertstetter et al. 2015) (Fig. 1 and Table 2). Studies using the membrane-permeable cCMP analog DB-cCMP showed that cCMP can trigger vasodilatation and inhibit platelet aggregation (Desch et al. 2010). In PKGI knockout mice, these findings were not observed any longer, indicating that cCMP can act via PKG. Recent findings indicated that cCMP mediates interactions with MAPK and regulates MAPK phosphorylation (Wolfertstetter et al. 2015) (see Sect. 4 for more details).

Other potential targets of cCMP are MRP5 (multidrug-resistance protein transporter) (Laue et al. 2014) and the HCN channels, cation channels, which can be modified by cyclic nucleotides. The HCN channels 2 and 4 can be activated by cCMP and cUMP and can be found particularly in the heart and in the brain where they affect the heartbeat rhythm, respectively (Zong et al. 2012). cCMP behaves as a partial agonist and accelerates the activation, but also slows down the deactivation of channels. Here cCMP acts directly on the HCN's own cNMP binding site in the C-terminus, which can be occupied also by cAMP or cGMP. The cNMP-binding domain of the HCN channels has a high sequence homology to the binding domain of PKGs. This may indicate that cCMP can also bind and act directly to the PKGs above the cNMP-binding domain. The HCN channels 1 and 3 are not sensitive to cCMP and cUMP (Zong et al. 2012).

The identification of potential substrates of cUMP is still arising. cUMP is able to activate HCN2 and HCN4 ion channels with low potency (Zong et al. 2012) (Fig. 2). Using membrane-permeable cUMP-AM, altered cellular activity was found with a DMR approach (Beckert et al. 2014b). These effects were only partly abolished by PKG or PKA inhibitors implying that other signaling pathways could be engaged by cUMP. Furthermore, gene expression of few genes related to cell cycle, differentiation, and stress was altered by cUMP-AM in B103 cells as shown by microarray analysis. Recent results using cUMP-agarose affinity chromatography indicated that besides PKG and PKA regulatory subunits, the PKA-anchoring proteins AKAP9, calnexin, and myomegalin exhibit affinity to cUMP. The functional implication of cUMP binding to these proteins is still in progress (Schneider and Seifert 2015). As ExoY exposure prominently enhances cUMP concentrations in lung tissue, it is conceivable that cUMP substrate proteins exist which might account for effects of this secreted factor from *Pseudomonas aeruginosa* (Beckert et al. 2014b; Schneider and Seifert 2015).

Table 2 Substrates for cCMP and/or cUMP

Substrate	cCMP binding	cUMP binding	Reference
PKARI/ PKARII	+	+	Hammerschmidt et al. (2012); Wolfertstetter et al. (2015); Wolter et al. (2011)
PKGI	+	+	Desch et al. (2010); Hammerschmidt et al. (2012); Wolfertstetter et al. (2015); Wolter et al. (2011)
PKGII	+	n.d.	Wolfertstetter et al. (2015)
PDE7A1	+ (Hydrolysis)	n.d.	Monzel et al. (2014)
PDE3A/ PDE3B/ PDE9A	n.d.	+ (Hydrolysis)	Reinecke et al. (2011)
HCN2	+	+	Zong et al. (2012)
HCN4	+	+	Akimoto et al. (2014); Zong et al. (2012)
IRAG	Activated by PKGIβ	n.d.	Desch et al. (2010)
MAPK	Activated by PKA, PKGI, PKGII (jejunum, lung)	n.d.	Wolfertstetter et al. (2015)
Rab23	+ (Rat brain)	n.d.	Bond et al. (2007)
MRP4	+	+	Laue et al. (2014)
MRP5	+	–	Laue et al. (2014)

n.d. not detected

4 Cross Talk Between cNMPs

Specificity of cyclic nucleotide signaling is regulated at the level of synthesis, substrate protein binding, transcellular transport, and/or degradation. Specific signaling pathways exist for cGMP or cAMP. However, the specificity of these pathways is challenged because at several of these regulatory mechanisms a possible cross talk between cNMPs has been elucidated. A summary of these analyses which engage cCMP and/or cUMP will be given below.

4.1 Cyclases

Synthesis of the cyclic nucleotides cGMP and/or cAMP is mediated by various different particular or soluble cyclases, namely, pGC, pAC, sGC, or sAC. pGC and AC have no activity for cCMP and/or cUMP synthesis. sGC and sAC are capable to generate cCMP and/or cUMP (Beste et al. 2012; Hasan et al. 2014). The synthesis of these cyclic nucleotides by sAC is stimulated by bicarbonate (Hasan et al. 2014). It is not clear whether the synthesis of these cyclic nucleotides by the cyclases sAC and sGC reaches physiological concentrations. Expression pattern of these cyclases

was compared with cNMP levels. Interestingly, as the high concentration of cCMP in some organs (e.g., spleen, pancreas, and FRS (female reproductive system)) does not correlate with sAC and sGC expression, it is assumed that specific synthases exist for this cyclic nucleotide (Bähre et al. 2015). Synthesis of cUMP is massively induced by ExoY form *Pseudomonas aeruginosa*. It is supposed that cUMP accounts for pathophysiological defects by *P. aeruginosa* infections which could engage cross talk or specific signaling pathways which have to be elucidated in the future.

4.2 Substrate Proteins

4.2.1 PKG and PKA

cCMP/cUMP are low-potency and low-efficacy activators of protein kinases A and G (Desch et al. 2010; Wolfertstetter et al. 2015; Wolter et al. 2011). The actions of cCMP on PKG in vivo lead to physiological implications in smooth muscle relaxation and inhibition of platelet aggregation (Desch et al. 2010). Interestingly, there are differences between in vitro and in vivo activation by cCMP analogs DB-cCMP and 4-MB-cCMP (4-monobutyryl-cCMP) on PKG and/or PKA so that a possible cross talk of these kinases could account for this result (Wolter et al. 2014). However, abolished DB-cCMP responses in PKGI knockout tissues, but not in PKA inactive cells, suggest a minor effect of cross talk under in vivo conditions. It will be of interest whether cUMP exerts also some specific functions due its interaction with PKA and/or PKG. There are indications that cUMP has an important role in toxin effects, e.g., mimicking the effects of ExoY (Beckert et al. 2014b). DMR responses on B103 and HEK293 cells in the presence of cCMP-AM/cUMP-AM with PKA /PKG inhibitors imply that cCMP and cUMP use different signaling pathways (Beckert et al. 2014a).

4.2.2 HCN Channels HCN2 and HCN4

Cross talk of cCMP and cUMP could be also relevant for the activation of HCN channels as both cyclic nucleotides are low-potency activators for these HCN channels (Zong et al. 2012). Interestingly, cCMP and cUMP activate HCN2 and HCN4 but not HCN1 or HCN3. cCMP was identified as a partial agonist which leads to lower efficacy (E_{max}, 0.6) and reduced activation (K_a 30 μM) compared to cAMP (K_a 1 μM). cUMP modulated HCN2 and HCN4 similarly to cCMP. Correlating the crystal structures, it was revealed that the principal cCMP- and cAMP-binding residues of HCN2 or HCN4 are identical. A weaker interaction of Arg-632 might explain the partial agonistic activity of cCMP (Zong et al. 2012). Furthermore, comparing cAMP and cCMP binding by NMR identified that cCMP binds to the C-terminal apo-CBD (cAMP-binding domain) in a different, more tetramerization-incompetent conformation than cAMP (Akimoto et al. 2014).

4.2.3 MAPK

The multistaged MAP kinase signaling pathways are particularly involved in the proliferation, differentiation, apoptosis, and growth of cells. It is still not clearly understood how cNMP signaling pathways (e.g., the cAMP or cGMP pathway) interact with the MAPK pathways. cAMP, for example, is able to activate the transcription factor Elk-1 on the MAPK cascade (Vossler et al. 1997). Furthermore it is certain that some effects of NO (including apoptosis) are mediated by the MAPK pathway. NO/cGMP activate the p38 pathway, for example, in neutrophils and fibroblasts (Browning et al. 1999, 2000). However, this field of research is discussed controversially (Jiang et al. 2013; Karakhanova et al. 2014; Stork and Schmitt 2002). Wolfertstetter et al. showed via co-immunoprecipitation that PKG interacts with p44/42 MAPK and that MAPK specifically binds to the cCMP agarose (Wolfertstetter et al. 2015). A direct interaction between MAPK and cCMP is most unlikely, because MAPK does not contain a cNMP binding site. The researchers assumed that cCMP interacts via activation/binding of another kinase (e.g., PKG) with the MAPK pathway. These results raise the question whether cCMP has an effect in cell regulation or during cell growth. Interestingly, cCMP-AM leads to caspase-dependent apoptosis which indicates the role of cCMP in stress-induced cellular responses (Wolter et al. 2015). With respect to an interaction of cUMP with MAPK, no data exist yet.

4.3 Transporters

MRP5 and MRP4 are efflux transporters for cGMP and cAMP (Wielinga et al. 2003). Functionally, MRP5 (ABCC5) regulates the smooth muscle activity by modulation of cGMP levels in concert with PDE5 (Al-Shboul et al. 2013). However, the transporter could be involved in further regulatory functions as it exports not only the cyclic nucleotide cGMP. Recent results indicated that cellular export of cCMP by MRP5 (with high capacity and low affinity) but not by MRP4 contributes to cellular inactivation of this cyclic nucleotide (Laue et al. 2014). Conversely, ABCC5 and ABCC4 (MRP4) are able to transport cUMP, and therefore both proteins might exhibit pleiotropic functions regarding the levels of various cyclic nucleotides (Laue et al. 2014). Recent results were reported that PDE5 inhibitors reduce cGMP export mediated by the ATP-binding cassette transporter ABCC5. Interestingly, PDE5 and ABCC5 have similar cGMP affinity. Furthermore, ABCC5 binds cGMP with higher affinity than cAMP. These experiments were performed with erythrocyte inside-out vesicles. It would be interesting whether it is also found in a more physiological setting. Furthermore, it is conceivable that also the export of cCMP or cUMP is affected by these PDE5 inhibitors (Aronsen et al. 2014).

4.4 Degradation by PDEs

Several PDEs were implicated to degrade cUMP. PDE3a, PDE3b, and PDE9a belong to these classes. These enzymes also degrade further cNMPs such as cAMP, cGMP, cIMP, and cXMP (Reinecke et al. 2011). The degradation of cCMP was up to now only detected for PDE7A1 and, with a lower activity, for PDE6AB, whereas cCMP is resistant for degradation by other PDEs. This implicates that cCMP is raised in several cells because of lacking degradation upon synthesis. PDE7A1 and PDE6AB have no specific cCMP phosphodiesterases. PDE7A1 hydrolyzes cAMP with a lower V_{max} than cCMP and also degrades cTMP. PDE6AB hydrolyzes also cIMP, cAMP, and cGMP (Monzel et al. 2014).

Taken together, cyclic nucleotide specificity is questioned by the new approaches regarding the function of cCMP and cUMP. Cross talk of these cyclic nucleotides to diverse "specific" cyclic nucleotide-activated proteins is of potential interest in pathophysiological or activated conditions where the basal level of these new cyclic nucleotides is strongly enhanced. However, up to now the detected levels of these molecules are very low so that a cross talk under physiological conditions can be probably excluded.

5 Future Research Directions

There are diverse approaches evolving which might promote the identification of new and/or specific substrate proteins of the noncanonical cyclic nucleotides cCMP and cUMP. The availability of new cyclic nucleotide-coupled matrices is a promising approach for the analysis of cNMP-binding proteins. However, new affinity tools (e.g., using Caprotec-cleavable linkers, CCMS) should support these methods.

The most important step in the cCMP/cUMP field would be the detection of highly specific signaling pathways for these cyclic nucleotides. Therefore, the genetic search for the specific cyclic nucleotide-binding motifs in diverse organisms, e.g., in mammalian and zebra fish, would be an important tool to evolve this emerging research field.

New cellular activity assays (e.g., employing the DMR method or new cell-permeable analogs) are also conceivable for the identification of functional cellular activities and pathways modified by the cyclic nucleotides.

The identification of cCMP and/or cUMP levels is up to now dependent on highly sophisticated methods employing mass spectrometry. To enlarge the applicability of the cyclic nucleotide detection, it would be of interest to generate specific antibodies which would be the base for a new cyclic nucleotide-based ELISA.

Some possible therapeutic options were discussed in a recent review (Seifert 2015). The most promising approach regarding cUMP signaling is the interfering in the ExoY activity of *Pseudomonas aeruginosa* infections. The response of cCMP on smooth muscle and platelets might lead to the applicability of stimulators of

these pathways in the cardiovascular system. Furthermore, the arising field of PDE inhibitors should envisage that the new cyclic nucleotides might also account for the effects of these molecules.

6 Conclusions

All these techniques which were described in this review provide valuable information and contribute to the present knowledge of the noncanonical second messengers cCMP and cUMP and their role in complex signaling pathways. The significance and impact of these noncanonical cyclic nucleotides strongly depend on the specific signaling targets. Therefore, research in this field should be of particular interest and might elucidate the possible (patho)physiological role and pharmacological applicability of cCMP and cUMP.

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Inactivation of Non-canonical Cyclic Nucleotides: Hydrolysis and Transport

Erich H. Schneider and Roland Seifert

Contents

Very recently, the first detailed analysis of enzyme kinetics was reported for cUMP hydrolysis by a class I phosphodiesterase, PDE3A (Berrisch et al. 2016). The hydrolysis of cUMP occurs with a K_M value of 143 μM, which is about 200-fold higher than the K_M value for cAMP (0.7 μM, determined under the same conditions). Moreover, the V_{max} for cUMP hydrolysis is 42 μmol/min/mg, which is more than 30-fold faster than the V_{max} reached by the cAMP-saturated enzyme (1.2 μmol/min/mg, determined under the same conditions) (Berrisch et al. 2016). Thus, PDE3A is a low affinity and high capacity PDE for cUMP. The PDE3 inhibitor milrinone reduced PDE3A-mediated cUMP hydrolysis with a K_i value of 57 nM (Berrisch et al. 2016), which is in good agreement with the K_i value reported in the literature for milrinone inhibition of cAMP hydrolysis (150 nM) (Ito et al. 1988). Thus, cUMP may bind to the same site as cAMP. Nevertheless, first experiments with HL-1 cardiomyogenic cells suggest that PDE-independent mechanisms are more important than PDE3 for the disposal of low intracellular cUMP concentrations (Berrisch et al. 2016). PDE3 may, however, prevent the toxic effects of cUMP under pathophysiological conditions (e.g. presence of cUMP-synthesizing bacterial nucleotidyl cyclases like ExoY).

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Abstract

This chapter addresses cNMP hydrolysis by phosphodiesterases (PDEs) and export by multidrug resistance associated proteins (MRPs). Both mechanisms are well-established for the canonical cNMPs, cAMP, and cGMP. Increasing evidence shows that non-canonical cNMPs (specifically cCMP, cUMP) are also PDE and MRP substrates. Hydrolysis of cUMP is achieved by PDE 3A, 3B, and 9A, which possibly explains the cUMP-degrading activities previously reported for heart, adipose tissue, and brain. Regarding cCMP, the only known "conventional" (class I) PDE that hydrolyzes cCMP is PDE7A. Older reports describe cCMP-degrading PDE-like activities in mammalian tissues, bacteria, and plants, but the molecular identity of these enzymes is not clear. High K_M and V_{max} values, insensitivity to common inhibitors, and unusually broad substrate specificities indicate that these activities probably do not represent class I PDEs. Moreover, the older results have to be interpreted with caution, since the historical analytical methods were not as reliable as modern highly sensitive and specific techniques like HPLC-MS/MS. Besides PDEs, the transporters MRP4 and 5 are of major importance for cAMP and cGMP disposal. Additionally, both MRPs also export cUMP, while cCMP is only exported by MRP5. Much less data are available for the non-canonical cNMPs, cIMP, cXMP, and cTMP. None of these cNMPs has been examined as MRP substrate. It was shown, however, that they are hydrolyzed by several conventional class I PDEs. Finally, this chapter reveals that there are still large gaps in our knowledge about PDE and MRP activities for canonical and non-canonical cNMPs. Future research should perform a comprehensive characterization of the known PDEs and MRPs with the physiologically most important cNMP substrates.

Keywords

cCMP • cUMP • Multifunctional PDE • Non-canonical cyclic nucleotides • Phosphodiesterases

1 Introduction to "Conventional" (Class I) PDEs and Their Regulation by cNMPs

A prerequisite for effective intra- and intercellular communication is the capability of cells to regulate intensity and duration of signal transduction processes. Therefore, inactivation of second messengers is essential and achieved by either transport mechanisms (outward transport into the extracellular environment or uptake by intracellular organelles) or enzymatic degradation. This chapter addresses hydrolytic inactivation of the non-canonical cNMPs, cIMP, cCMP, and cUMP and shortly discusses transporter-mediated cNMP disposal.

1.1 Traditional Classification of PDEs

Historically, the first cyclic nucleotide second messenger discovered was cAMP (Rall and Sutherland 1958), followed by reports about biological activity of cGMP (Hardman et al. 1966). Research in the subsequent decades focused on these two purine cNMPs. Therefore, cAMP and cGMP are considered "canonical" cNMPs (Fig. 1) (Seifert et al. 2015), while research on "non-canonical" (Fig. 1) (Seifert et al. 2015) cNMPs, like the cyclic purine nucleotides cIMP and cXMP as well as the cyclic pyrimidine nucleotides cCMP, cUMP, and cTMP, was neglected for a long time. This may have contributed to the current cAMP- and cGMP-centered classification of PDEs.

Phosphodiesterases form eleven isoform families (PDE1-11) that are classified into three groups according to their specificity for the canonical cyclic nucleotides cAMP and cGMP (Bender and Beavo 2006; Azevedo et al. 2014). All members of these eleven PDE families hydrolyze the phosphodiester bond of 3′,5′-cNMPs, yielding 5′-NMPs. To the best of our knowledge there are no reports suggesting hydrolysis of 2′,3′-cNMPs or formation of 3′-NMPs by any of these PDEs. The PDE families 4, 7, and 8 prefer cAMP, while PDE5, 6, and 9 are cGMP-specific (Fig. 2).

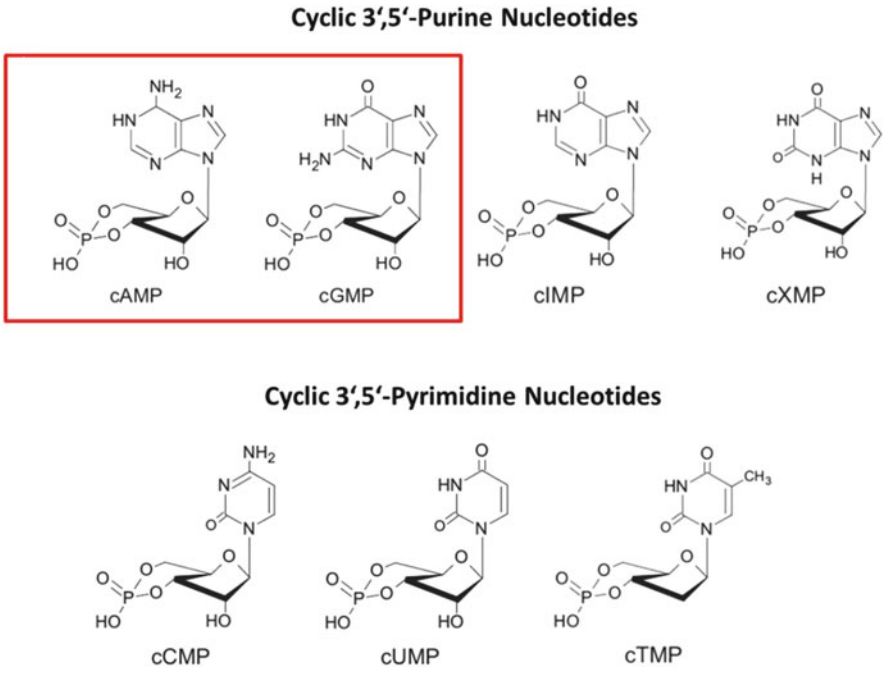

Fig. 1 Chemical structures of the canonical purine cNMPs (*red box*, cAMP and cGMP) and of the non-canonical purine (cIMP, cXMP) and pyrimidine (cCMP, cUMP, and cTMP) cNMPs. Adapted from Beste and Seifert (2013)

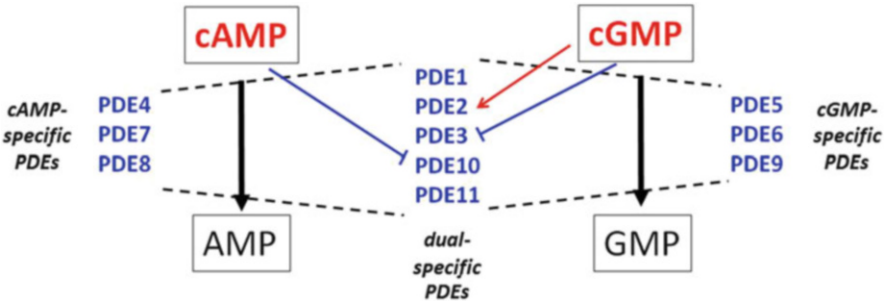

Fig. 2 Classification of PDEs according to their specificity for the canonical cyclic nucleotides cAMP and cGMP. In addition, three examples for modulation of PDE activity by cNMPs are shown

The PDEs 1, 2, 3, 10, and 11 are named "dual-specific," because they hydrolyze both cAMP and cGMP with comparable K_M values (Fig. 2).

1.2 Direct Regulation of PDE Activity by cNMPs

Fine-tuning of cNMP signaling is achieved by modulation of PDE activity. In addition to indirect ways like regulation of PDE expression, PDE activity is also directly modified by intracellular activating or inhibiting factors. In this regard, specifically cNMPs are of major importance. Regulation of PDE activity by cNMPs can occur in two ways, namely (1) by direct binding to the catalytic center of the enzyme or (2) by interaction with cNMP-binding regulatory domains, the so-called GAF domains. The abbreviation "GAF" is derived from the names of the three proteins, where this kind of domain was first identified, namely c**G**MP-specific and cGMP-stimulated phosphodiesterases, *Anabaena* **a**denylyl cyclases and *Escherichia coli* **F**hlA domains.

An example for the first case is PDE3, which binds both cAMP and cGMP with affinity in the nanomolar range, but exhibits an about 10-times higher V_{max} value for cAMP as compared to cGMP (Bender and Beavo 2006). Consequently, PDE3 can be considered a cGMP-inhibited cAMP PDE (Fig. 2). On the contrary, PDE10 binds and hydrolyzes cAMP with higher affinity and 2–5-fold lower V_{max} as compared to cGMP and has therefore properties of a cAMP-inhibited cGMP PDE (Fig. 2) (Bender and Beavo 2006).

Another possibility of regulation of PDE activity is binding of cNMPs to the GAF domains, which occur twice per molecule (GAF-A and GAF-B) in PDEs 2, 5, 6, 10, and 11 (Azevedo et al. 2014). Binding of cGMP to the GAF-B domain of PDE2 activates PDE2-mediated cAMP hydrolysis (Fig. 2) (Bender and Beavo 2006; Beavo et al. 1971; Jäger et al. 2010). Binding of cGMP to the PDE5 GAF-A domain increases catalytic activity of the enzyme (Bender and Beavo 2006; Zoraghi et al. 2005; Rybalkin et al. 2003). GAF domain-mediated regulation

of catalytic activity in PDE10 is characterized by two uncommon features. First, the PDE10 GAF domain tandem binds cAMP with higher affinity than cGMP (Jäger et al. 2012), which indicates that GAF domains are not necessarily cGMP-specific. Second, although cAMP binding to PDE10 GAF-B enhances catalytic activity for cGMP hydrolysis at low concentrations, cAMP concentrations higher than 0.1 μM completely inhibit cGMP hydrolysis (Jäger et al. 2012). This indicates that PDE10 is only "dual-specific" at very low cAMP concentrations, but is converted to a cAMP-specific enzyme at cAMP concentrations >0.1 μM (Jäger et al. 2012). Finally, in case of the PDE11 GAF domains, the naturally occurring stimulating ligand is still unknown. Although cGMP bound to FRET constructs of PDE11 GAF domains with relatively high affinity, it was not capable of stimulating the holoenzyme (Jäger et al. 2012).

The contribution of non-canonical cyclic nucleotides to GAF domain-mediated regulation of PDE activity is largely unexplored. The pyrimidine cyclic nucleotides cUMP and cCMP were basically inactive at a FRET construct of PDE10 GAF domains (Jäger et al. 2012) and did not activate a chimeric protein consisting of PDE10 GAF domains and cyanobacterial cyclase (Gross-Langenhoff et al. 2006). The non-canonical cyclic purine nucleotide cIMP could not activate PDE5-mediated hydrolysis of the fluorescent cGMP analogue 2′-*O*-anthraniloyl cGMP, while cGMP stimulated PDE5 activity (Okada and Asakawa 2002). Moreover, cIMP was inactive at a fusion protein of PDE11A4 GAF domains with cyanobacterial cyclase (Gross-Langenhoff et al. 2006). The corresponding chimeric protein of PDE10 GAF domains and bacterial cyclase was only slightly activated (~60%) by 100 μM of cIMP, suggesting that cIMP is not regulating PDE10 (Gross-Langenhoff et al. 2006). By contrast, cIMP bound with submicromolar affinity to the GAF domains of PDE2 and PDE5 (Jäger et al. 2010). The only GAF domain known so far that prefers cCMP over cAMP and cGMP is part of the protein RSP2 (radial spoke protein 2) from the green alga *Chlamydomonas reinhardtii* (Yao and Sem 2005). Interestingly, as discussed below, the same organism also contains a cCMP-hydrolyzing PDE activity, which, however, exhibits only very low cCMP affinity in the millimolar range (Fischer and Amrhein 1974).

2 PDE-Mediated Hydrolysis of Non-Canonical cNMPs

Several literature reports describe cCMP-, cUMP-, and cIMP-hydrolyzing activities in mammalian cells. Most of the publications, however, do not provide a molecular identification of these activities in terms of defined PDE isoforms, although some papers report purification down to the level of a single protein. In the following, the available literature on PDE activities for non-canonical cNMPs will be summarized and evaluated. Moreover, an overview of cCMP-degrading enzymes is provided for mammalian cells in Table 1 and for plants and bacteria in Table 2. Unspecific "multifunctional" PDEs are listed in Table 3 and cUMP-hydrolyzing enzymes are shown in Table 4. For cIMP, cXMP, and cTMP degradation, no separate table is provided, since literature on PDE-mediated hydrolysis of these cNMPs is very limited.

Table 1 High K_M cCMP PDE in mammalian cells

Tissue preparation, species	K_M (mM); V_{max} (nmol/min/mg)[a]				Inhibitors (examples)	Activators	Mol. mass (kDa)	pH optimum	References
	cAMP	cGMP	cCMP	cUMP					
L1210 mouse leukemia cells	K_M: n.d. $V_{1\ mM}$: 0	K_M: n.d. $V_{1\ mM}$: 0	K_M: n.d. $V_{1\ mM}$: 58,000	n.d.	n.d.	$MgSO_4$ $(NH_4)_2SO_4$	n.d.	7–8	Cheng and Bloch (1978)
Rat liver, other rat and guinea pig organs	K_M: n.d. $V_{1\ mM}$: 294[b]	K_M: n.d. $V_{1\ mM}$: 244[b]	K_M: 2.4 $V_{1\ mM}$: 1,092[b]	n.d.	3′,5′-cNMPs (U, A,G,I); 2′-deoxy-cNMPs (G, A); no effect of IBMX	At 10 mM: $Fe^{2+} > Mn^{2+} > Mg^{2+}$	n.d.	6.5–7.5	Kuo et al. (1978)
Rat liver	K_M: 11.2 V_{max}: 290	K_M: 14.7 V_{max}: 160	K_M: 9.0 V_{max}: 48,600	K_M: 8.7 V_{max}: 92	n.d.	Fe^{2+} added to PDE assay samples	28	7.2–7.4	Newton and Salih (1986)
Rat liver	K_M: 12.4[c] V_{max}: 230[c]	K_M: 15.8[c] V_{max}: 180[c]	K_M: 7.3[c] V_{max}: 53,200[c]	K_M: 9.7[c] V_{max}: 110[c]	n.d.	Fe^{2+} added to all PDE-assay samples	n.d.	n.d.	Newton et al. (1999)
Adult guinea pig liver homogenates[d]	K_M: n.d. $V_{1\ mM}$: 0.298	K_M: n.d. $V_{1\ mM}$: 0.744	K_M: n.d. $V_{1\ mM}$: 0.127	n.d.	n.d.	10 mM Fe^{2+} added to all PDE-assay samples[e]	n.d.	n.d.	Shoji et al. (1978a)
Rat liver homogenate[f]	K_M: n.d. $V_{1\ \mu M}$: 0.062	K_M: n.d. $V_{1\ \mu M}$: 0.033	K_M: 4.2 $V_{1\ \mu M}$: 0.00111 $V_{1\ mM}$: 0.601	n.d.	n.d.	10 mM Fe^{2+} added to all PDE-assay samples	n.d.	n.d.	Shoji et al. (1978b)

Rat liver homogenate[f]	K_M: n.d. V_{max}: n.d.	K_M: n.d. V_{max}: n.d.	K_M: 2.85 V_{max}: 1.8	n.d.	Zn^{2+}; >5 mM of Fe^{2+}	>5 mM: $La^{3+} \sim Mn^{2+} > Mg^{2+} > Ca^{2+}$; maximum activation by Fe^{2+} at 5 mM	n.d.	~7.0	Wei and Hickie (1983)

n.d. not determined

[a]V is either given as V_{max} or as V at a specific substrate concentration (e.g., $V_{1\ mM}$), dependent on the available data

[b]Values are given as nmol/min; no exact amount of enzyme protein is given (range: 215–405 μg)

[c]From radiometric assays

[d]For differences between fetal and adult tissues and for cCMP PDE in regenerating tissues see Sect. 2.1.1 of the text

[e]Addition of Fe^{2+} was only explicitly stated for PDE assays with rat tissues, but the assays with guinea pig livers were most likely conducted under the same conditions

[f]This paper also reports on the relationship between the growth rate of Morris hepatoma and cCMP PDE activity, which is not considered in this table (see Sect. 2.1.1)

Table 2 High and intermediate K_M cCMP-degrading activities in plants and bacteria

Tissue preparation, species	K_M (mM); V (nmol/min/mg)[a]				Inhibitors (examples)	Activators (examples)	Mol. mass (kDa)	pH optimum	References
	cAMP	cGMP	cCMP	cUMP					
Preparation from *Serratia marcescens*	K_M: 0.52 $V_{5\ mM}$: 100%[b] V_{max}: 295,000	K_M: n.d. $V_{5\ mM}$: 52.2%[b]	K_M: 20 $V_{5\ mM}$: 17.4%[b]	K_M: n.d. $V_{5\ mM}$: 51.7%[b]	Theophylline, proflavine, 8-hydroxy-quinoline, cyclic phosphonate[c], Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}	Fe^{2+}, Ca^{2+}, Ba^{2+}	51	7.5–8.5	Okabayashi and Ide (1970)
Extracts of *Chlamydomonas reinhardtii*	K_M: 0.085 $V_{2\ mM}$: 100%[b]	K_M: n.d. $V_{2\ mM}$: 50%[b]	K_M: n.d. $V_{2\ mM}$: 300%[b]	K_M: n.d. $V_{2\ mM}$: 30%[b]	Methylxanthines, pyrophosphate, papaverine, Zn^{2+}, Co^{2+}	Imidazole, cysteine, Mn^{2+}, Mg^{2+}	n.d.	8.5	Fischer and Amrhein (1974)
Pisum sativum pea seedling extract	K_M: 0.9 $V_{4\ mM}$: 41%[b]	K_M: 1.61 $V_{4\ mM}$: 26%[b]	K_M: 1.81 $V_{4\ mM}$: 23%[b]	K_M: 0.58 $V_{4\ mM}$: 68%[b]	NaF (only 3′,5′-cNMP hydrolysis), P_i, PP_i, various nucleotides	Cysteine, dithiothreitol, slight effect of Mn^{2+}, Co^{2+} and Zn^{2+}	350	5.4–6.0	Lin and Varner (1972)
Pisum sativum seedling extract (50–75%)[d]	K_M: n.d. V: 3.6[e]	K_M: n.d. V: 1.9[e]	K_M: n.d. V: 9.4[e]	n.d.	n.d.	n.d.	n.d.	n.d.	Chiatante et al. (1990)
Pisum sativum seedling extract (75–100%)[d]	K_M: n.d. V: 2.1[g]	K_M: n.d. V: 0[g]	K_M: 0.5[f] V_{max}: 122[f] V: 31.3[g]		Theophylline[f], EDTA[f]	Mg^{2+}; Ca^{2+} and Fe^{3+} synergistic[f]			
Lactuca sativa extracts	K_M: 1.1 V_{max}: 5,700	K_M: 0.71 V_{max}: 2,600	K_M: 0.64 V_{max}: 2,200	n.d.	Zn^{2+}, Cu^{2+}	Fe^{3+}, Mn^{2+}, Co^{2+}, Cr^{3+}	62	n.d.	Chiatante et al. (1986)

Lactuca sativa extracts[h]	K_M: 0.93 V_{max}: 312.1	K_M: 0.73 V_{max}: 110.9	K_M: 0.67 V_{max}: 221.0	n.d.	cNMPs inhibit each other[i]	n.d.	61–63	5.5	Chiatante et al. (1987)
Phaseolus vulgaris extracts[j]	K_M: n.d. $V_{2\ mM}$: 100[k]	K_M: n.d. $V_{2\ mM}$: 95[k]	K_M: n.d. $V_{2\ mM}$: 79[k]	K_M: n.d. $V_{2\ mM}$: 91[k]	Slight inhibition by 5 mM of caffeine and theophylline	in the presence of fraction G: Ca^{2+}, abscisic acid[l]	34	6.5	Brown et al. (1977)
Solanum tuberosum potato tuber extract	K_M: 1.5 $V_{5\ mM}$: 15%[m]	K_M: n.d. $V_{5\ mM}$: 12%[m]	K_M: n.d. $V_{5\ mM}$: 15%[m]	n.d.	Phosphate?	n.d.	79–81	5.5–6.5	Zan-Kowalczewska et al. (1984)
Solanum tuberosum potato tuber extract	K_M: 1.5 $V_{5\ mM}$: 2%[n]	After heating to 45°C at pH 8.8: 3′,5′-cNMP hydrolysis eliminated; 2′,3′-cNMP hydrolysis unaffected; molecular mass unchanged; pH optimum left-shifted for p-nitrophenyl phenylphosphonate, but not for cNMPs							Zan-Kowalczewska et al. (1987)

n.d. not determined

[a]V is either given as V_{max} or as V at a specific substrate concentration (e.g., $V_{1\ mM}$) or as relative rate, dependent on the reported data

[b]Okabayashi and Ide (1970), Fischer and Amrhein (1974): relative reaction speed in % of 3′,5′-cAMP hydrolysis; Lin and Varner (1972): relative reaction speed in % of 2′,3′-cUMP hydrolysis; the enzymes described by Okabayashi and Ide (1970), Fischer and Amrhein (1974) and Lin and Varner (1972) also accept 3′,5′-cIMP and -cTMP

[c]Cyclic phosphonate = 5′-deoxy-5′-(dihydroxyphosphinylmethyl)-adenosine-3′-cyclic ester

[d]$(NH_4)_2SO_4$ fractionation

[e]Determined with the crude 50–75% fraction from meristematic tissue; substrate concentration unclear

[f]Determined with affinity-purified enzyme (Affi-Gel Blue)

[g]Determined with the crude 75–100% fraction from meristematic tissue; substrate concentration unclear

[h]Hydrolysis of 2′,3′-cNMPs was also analyzed, but K_M and V_{max} values are not shown in this table

[i]Probably more than one binding site available for each nucleotide

[j]Only data for peak 1 from the Sephadex G-200 purification are shown in this table; peak 2 exhibited similar PDE activity, but a molecular mass of only 7.6 kDa

[k]This is the relative reaction speed in % of 3′,5′-cAMP hydrolysis; the enzyme also accepted cIMP, cXMP, and cdTMP (not shown in table)

[l]Ca^{2+} or abscisic acid acted stimulatory, when they were combined with fraction G (= pooled post-peak 1 fractions) of the Sephadex G-200 purification step

[m]This is the relative reaction speed in % of 2′,3′-cAMP hydrolysis; the enzyme also accepts 2′,3′-cNMPs (not shown in table)

[n]Relative reaction speed in % of 2′,3′-cAMP hydrolysis, determined with "inactivated" enzyme (see text in table)

Table 3 Intermediate K_M multifunctional PDE in mammalian cells

Tissue preparation, species	K_M (μM); V (nmol/min/mg)[a]				Inhibitors (examples)	Activators	Mol. mass (kDa)	pH optimum	References
	3′,5′-cAMP	3′,5′-cGMP	3′,5′-cCMP	3′,5′-cUMP					
Pig liver extract	K_M: 25 V_{max}: 1,600	n.d.[b]	K_M: 182 V_{max}: 4,100	n.d.	Co^{2+}, Fe^{2+}, Zn^{2+}	Slight stimulation by Mg^{2+}, Ca^{2+} and Mn^{2+}	31–37 (globular)	7.0	Helfman et al. (1981)
Pig liver extract	Same enzyme as reported by Helfman et al. (1981). This paper reports hydrolysis of both 3′,5′- and 2′,3′-cNMPs (C,U,G,A)				3′,5′/2′,3′-cCMP hydrolysis inhibited by 2′,3′/3′,5′-cAMP	n.d.	n.d.	n.d.	Helfman and Kuo (1982a)
Pig liver extract	Detailed characterization of inhibitor profile of the enzyme reported by Helfman et al. (1981). Resistant to papaverine, IBMX, theophylline, and caffeine; inhibited by 5′-CMP, UMP, AMP, and GMP and to a lesser extent by di- and trinucleotides (NMP > NDP > NTP); strong inhibition by phosphate								Helfman and Kuo (1982b)
Rat liver extract	K_M: 25 V_{max}: n.d.	K_M: 237 V_{max}: n.d.	n.d.[c]	n.d.	Insensitive to IBMX and numerous other PDE inhibitors	No effect of Mg^{2+} or Ca^{2+}/Calmodulin	33	n.d.	Lavan et al. (1989)
Rat liver extract	K_M: 47 V_{max}: n.d.[d]	K_M: >500 V_{max}: n.d.[d]	K_M: >100 V_{max}: n.d.[d]	n.d.[d]	IBMX-insensitive; inhibited by AMP and phosphate	No effect of Mg^{2+} or Ca^{2+}/Calmodulin	25	n.d.	Worby et al. (1991)

Human liver extract	K_M: 23 V_{max}: 1.16[e]	n.d.	K_M: 75 V_{max}: 2.32[e]	n.d.	IBMX-insensitive; inhibited by phosphate	n.d.	38 (globular)	n.d.	Mendel et al. (1997)
Rat or pig liver extract?[f,g]	K_M: 38.3[h] V_{max}: 5,800[h]	K_M: 482[h] V_{max}: 3,600[h]	K_M: 132[h] V_{max}: 14,300[h]	K_M: 53.2[h] V_{max}: 6,300[h]	cNMPs inhibit each other's hydrolysis	n.d.	n.d.	n.d.	Newton et al. (1999)

n.d. not determined

[a]V is either given as V_{max} or as V at a specific substrate concentration (e.g., $V_{1\ mM}$), dependent on the available data

[b]cGMP is also hydrolyzed. The specificity profile is cAMP > cCMP > cGMP at 1 μM of substrate and cCMP > cAMP > cGMP at 1 mM

[c]cCMP-hydrolysis was not determined by Lavan et al. (1989), but it was suggested by Worby et al. (1991) that this enzyme may have been the multifunctional PDE

[d]The enzyme hydrolyzes both 2′,3′- and 3′,5-cNMPs. Hydrolysis rates at 2 mM: cCMP > cAMP > cGMP (3′5′-cNMPs) and cCMP > cGMP > cAMP (2′,3′-cNMPs)

[e]V_{max} values are not related to mg, but to "unit" (amount of enzyme converting 1 nmol/h of cHPMPC to cidofovir at 37°C and at a substrate concentration of 10 μM)

[f]After describing the preparation of cCMP-specific PDE from rat liver, the authors write: "A sample of the multifunctional phosphodiesterase was similarly produced from an analogous homogenate by the method described by Helfman." Helfman, however, prepared the multifunctional PDE from pig liver. Thus, it is not clear if Newton et al. (1999) have used rat or pig liver for the preparation of multifunctional PDE

[g]Hydrolysis of 2′,3′-cNMPs was also analyzed, but K_M and V_{max} values are not shown in this table

[h]From radiometric assays

Table 4 cUMP-hydrolyzing enzymes

Tissue preparation, species	K_M (mM); V_{max} (nmol/min/mg)[a]				Inhibitors (examples)	Activators (examples)	Mol. mass (kDa)	pH optimum	References
	cAMP	cGMP	cCMP	cUMP					
Rabbit brain homogenate	$V_?$: 100%[b]	$V_?$: 33%[b]	No hydrolysis[b]	$V_?$: 11%[b]	inhibited by >0.8 mM of Mg^{2+}	Mg^{2+} required	n.d.	>7[c]	Drummond and Perrott-Yee (1961)
Dog heart homogenates	$V_{360\ \mu M}$: 170[d]	<25% of cUMP hydrolysis rate	<5% of cUMP hydrolysis rate	$V_{360\ \mu M}$: 491[d]	theophylline[e], caffeine[e]	Mg^{2+} required; imidazole[f]	n.d.	~8	Hardman and Sutherland (1965)
Dog heart homogenates	K_M: n.d. $V_{2.1\ mM}$: 100%	K_M: n.d. $V_{2.1\ mM}$: 33%	No hydrolysis	K_M: n.d. $V_{2.1\ mM}$: 12–15%	caffeine, >1 mM of Mn^{2+} or Co^{2+}	Mg^{2+} required, ammonium salts, imidazole	n.d.	8.5–9.2	Nair (1966)
Rat brain cortex	K_M: 0.1–0.3 $V_{2\ mM}$: 100%	K_M: n.d. $V_{2\ mM}$: 70%	n.d.	K_M: n.d. $V_{2\ mM}$: 10%	Ca^{2+}, Cu^{2+}, Zn^{2+} theophylline, caffeine, NTPs	Mg^{2+} required, ammonium, imidazole	n.d.	~8	Cheung (1967)
Rat adipose tissue	K_M: 0.1 V_{max}: 5.7	n.d.	n.d.	K_M: 0.58 V_{max}: 11.4	theophylline, caffeine, papaverine	n.d.	n.d.	n.d.	Klotz and Stock (1971)

PDE3A	$V_{10\ \mu M}$: 121	$V_{10\ \mu M}$: 20.3	No hydrolysis detected	$V_{10\ \mu M}$: 102	Only 3′,5′-cNMP hydrolysis was studied, using recombinant human enzymes with defined sequence and purity	Reinecke et al. (2011)
PDE3B	$V_{10\ \mu M}$: 162	$V_{10\ \mu M}$: 34.5		$V_{10\ \mu M}$: 50.4		
PDE9A	$V_{10\ \mu M}$: 30.5	$V_{10\ \mu M}$: 13.7		$V_{10\ \mu M}$: 36.6		

n.d. not determined

[a]If not otherwise indicated, V is either given as V_{max} or as V at a specific substrate concentration (e.g., $V_{1\ mM}$) or as relative rate

[b]Substrate concentration, at which V was determined, was not clearly stated; Fig. 1 of the paper suggests 0.13 mM

[c]The experimental method did only allow measurements in the narrow range between 5.5 and 7.5

[d]V is given as nmol/min/g wet weight

[e]Differential activity against cAMP and cUMP hydrolysis (may be caused by the presence of more than one PDE in the preparation)

[f]Only moderate stimulatory effect of imidazole on cUMP hydrolysis, but strong effect on cAMP hydrolysis

2.1 cCMP-Degrading Activities

Most of the cCMP PDEs described in the literature were isolated from liver tissue and can be divided essentially into two groups. One type of activity shows an extremely high K_M value in the millimolar range and is referred to as "cCMP PDE" (Newton et al. 1999). The other type of cCMP-degrading enzyme, which is named "multifunctional PDE," hydrolyzes cCMP with a K_M value of around 200 μM and accepts also other 3′,5′- and even 2′,3′ cyclic nucleotides (Newton et al. 1999). These two "classes" of cCMP-hydrolyzing activities will be described in more detail in the following sections.

2.1.1 Low-Affinity cCMP PDE in Mammalian Tissues

The first report on a high K_M "cCMP-specific" PDE activity was published more than 30 years ago (Cheng and Bloch 1978). Homogenates of L-1210 leukemia cells hydrolyzed cAMP, cGMP, and cCMP. Ammonium sulfate precipitation, however, yielded a fraction at 80–100% $(NH_4)_2SO_4$, which specifically degraded cCMP (Cheng and Bloch 1978). This cCMP-hydrolyzing activity was stimulated by $MgSO_4$ or $(NH_4)_2SO_4$ and became saturated only at cCMP concentrations higher than 10 mM (Cheng and Bloch 1978). In the same year, another group reported on a cCMP-preferring PDE activity in rat liver extract, which was enriched by pH fractionation with acetic acid (Kuo et al. 1978). Similar to the enzyme from L-1210 cells (Cheng and Bloch 1978), this activity had a very high K_M value for cCMP (2.4 mM). cCMP was hydrolyzed at almost 4-times the rate of cAMP at a substrate concentration of 1 mM (Kuo et al. 1978). The pH optimum of this activity was between 6.5 and 7.5. Surprisingly, cCMP hydrolysis was strongly stimulated in the presence of Fe^{2+} (10 mM) and to a lesser extent by millimolar concentrations of Mg^{2+} and Mn^{2+}. "Classical" PDE inhibitors like theophylline (2 mM) or IBMX (100 μM) were only slightly effective, while cAMP (1 mM) showed a strong inhibitory effect (Kuo et al. 1978). This low-affinity cCMP PDE was also detected in other organs, specifically in kidney and intestine (Kuo et al. 1978). Eight years later, this cCMP-specific PDE was purified down to the level of a single protein. It showed absolute specificity for cCMP, a cCMP K_M value of 9 mM, and a pH optimum of 7.2–7.4 (Newton and Salih 1986). The maximum cCMP-hydrolyzing activity was reached at a temperature of 32°C, and the enzyme was inactivated at 60°C (Newton and Salih 1986). A determination of amino acid composition revealed glutamate and aspartate as the two most common residues and a molecular mass of about 28 kDa (Newton and Salih 1986).

The activity of low-affinity cCMP PDE in various tissues seems to be inversely correlated to the extent of cell proliferation, because it was reduced in regenerating rat liver as compared to normal liver (Kuo et al. 1978; Shoji et al. 1978a). Moreover, cCMP hydrolysis was lower in liver and various other tissues of fetal guinea pigs as compared to the corresponding tissues from adult animals (Kuo et al. 1978; Helfman et al. 1978). Finally, as reported independently by two research groups, cCMP PDE activity was also depressed in fast-growing Morris hepatoma as compared to slow-growing tumors or normal liver (Shoji et al. 1978b; Wei and

Hickie 1983). The inverse correlation between cCMP PDE activity and tissue growth rate suggests a potential role of cCMP in the regulation of cell proliferation. In fact, cCMP reportedly promotes resumption of cell growth in L-1210 cell cultures, which had been arrested in the G1 phase by cooling them down to 4°C for 1 h (Bloch et al. 1974). Moreover, enhanced cCMP concentrations were detected in leukocytes and urine samples from patients with acute myeloblastic or lymphoblastic leukemia (Scavennec et al. 1981). These results, however, should be regarded with caution, since they were obtained with a radioimmunoassay for cCMP, which has limited sensitivity and specificity. Nevertheless, the presence of cCMP in cancer cell lines or immortalized cells has been demonstrated very recently by using modern HPLC-coupled tandem mass spectrometry methods (Hartwig et al. 2014). It would be interesting to look for a correlation between intracellular cCMP levels and cellular proliferation rate. Moreover, every type of cell may possess an individual "cNMP signature" (Hartwig et al. 2014). It remains to be elucidated, if the ratio between the various cNMPs is also important for the growth characteristics of a cell.

Taken together, the cCMP PDE seems to represent a rather "exotic" PDE-like activity, which does not belong to the established class I PDEs. This is suggested by a combination of properties not found in any of the conventional PDEs, namely (1) absolute cCMP specificity, (2) the millimolar K_M value, (3) the very low molecular mass of 28 kDa, (4) the stimulation by Fe^{2+}, and (5) the resistance to PDE inhibitors like IBMX or theophylline. It might be argued that it is not appropriate to call an enzyme cCMP-"specific," when the K_M value is in the millimolar range. In fact, data from a detailed characterization of rat liver cCMP PDE by mass spectrometry methods reveal that this enzyme hydrolyzes cCMP with a K_M of 7.3 mM, but cAMP, cGMP, and cUMP are also hydrolyzed with K_M values of 12.4, 15.8, and 9.7 mM, respectively (Newton et al. 1999). The V_{max} value of the cCMP PDE for cCMP, however, is at least 150-fold higher than for any other of the tested cNMPs (Newton et al. 1999). Thus, the observed cCMP-"specificity" of cCMP PDE is not a result of higher cCMP affinity as compared to other cNMPs, but rather caused by an extremely high V_{max} value. In summary, cCMP PDE is a low-affinity but high-velocity enzyme for cCMP. At the moment, however, it is not clear yet if the cCMP-degrading activity of cCMP PDE is also important under physiological conditions. Specifically the high K_M value suggests that it may be a "bystander activity" of an enzyme that normally hydrolyses completely different and as yet unknown substrates. It should also be noted that the 28 kDa protein identified as "cCMP PDE" may have resulted from proteolytic degradation of a larger enzyme. Moreover, cCMP PDE may form oligomers with distinct properties under physiological conditions, as it was described previously for PDE4 (Richter and Conti 2004).

2.1.2 Multifunctional PDE in Mammalian Tissues

The second type of cCMP-hydrolyzing activity shows a K_M value in the range of 100–200 μM and was first isolated from pig liver extract by pH and $(NH_4)_2SO_4$ fractionation, followed by several chromatography procedures (Helfman et al.

1981). The purification procedure yielded a single protein with a molecular mass of ~33 kDa (SDS-PAGE). This molecular mass was confirmed by gel filtration and linear sucrose density gradient ultracentrifugation, revealing 31 kDa and 36.5 kDa, respectively (Helfman et al. 1981). The enzyme seems to be a globular protein, and the isoelectric point of pH 4.6 indicates acidic character. In fact, the analysis of amino acid composition revealed that about ~25% of the protein consists of the acidic amino acids aspartate and glutamate (Helfman et al. 1981). The pH optimum of this PDE was ~pH 7.0. The activity was Ca^{2+}/calmodulin independent, but slightly stimulated by Mg^{2+}, Ca^{2+}, and Mn^{2+}. By contrast, Co^{2+}, Fe^{2+}, and Zn^{2+} were inhibitory (Helfman et al. 1981). Most interestingly, this enzyme showed no absolute substrate specificity, but hydrolyzed the cyclic purine nucleotides cAMP and cGMP as well as the cyclic pyrimidine nucleotide cCMP. The K_M values for cCMP and cAMP were 182 μM and 25 μM, respectively. The V_{max} value for cCMP hydrolysis (4.1 μmol/min/mg) was higher than for cAMP degradation (1.6 μmol/min/mg) (Helfman et al. 1981).

Shortly later, it was demonstrated that this enzyme also hydrolyzes 3′,5′-cUMP and does not even discriminate between 3′,5′- and 2′,3′ cyclic nucleotides. Therefore, it was named "multifunctional phosphodiesterase" (Helfman and Kuo 1982a). Interestingly, while hydrolysis of 3′,5′-cAMP, cGMP, cCMP, and cUMP consistently yielded 5′-NMPs as products, hydrolysis of the corresponding 2′,3′-cNMPs resulted in mixtures of 2′- and 3′-NMPs and the proportions of the 2′ and 3′ products depended on the nucleobase of the hydrolyzed cNMP (Helfman and Kuo 1982a). The range of hydrolysis rates was cCMP > cUMP > cGMP ≥ cAMP for 2′,3′-cNMPs and cCMP > cUMP ≥ cAMP > cGMP for 3′,5′-cNMPs (Helfman and Kuo 1982a). When 2′3′-cNMPs and 3′,5′-cNMPs with the same nucleobase were compared, the multifunctional PDE hydrolyzed the 2′,3′-cNMP faster than the 3′,5′-cNMP (Helfman and Kuo 1982a). The results of mixed substrate experiments suggest that this enzyme possesses a single catalytic site that accepts pyrimidine as well as purine 2′,3′- or 3′,5′-cNMPs (Helfman and Kuo 1982a).

When compared to the cCMP PDE discussed in the preceding section, the kinetic features of the multifunctional PDE (K_M and V_{max}) are much closer to conventional PDEs. Nevertheless, the substrate profile and the inhibitor sensitivity of the multifunctional PDE suggest that this enzyme does not belong to any group of established PDEs. Multifunctional PDE is practically insensitive to "classic" PDE inhibitors like papaverine, IBMX, theophylline, and caffeine (inhibition < 16%) (Helfman and Kuo 1982b). However, 1 mM of the nucleoside 5′-monophosphates CMP, UMP, AMP, and GMP reduced the activity of multifunctional PDE for 3′,5′-cCMP and 3′,5′-cAMP by >60% (Helfman and Kuo 1982b). Comparison of nucleotide mono-, di-, and tri-phosphate (1 mM) hydrolysis revealed that the inhibitory effect on multifunctional PDE activity decreases with increasing number of phosphate residues, resulting in the inhibitory rank order NMP > NDP > NTP (Helfman and Kuo 1982b).

The existence of a cCMP-degrading multifunctional PDE activity was further corroborated by independent findings of other research groups. A chromatographic analysis of rat liver PDEs revealed a Mg^{2+}-independent enzymatic activity that

hydrolyzed cAMP ($K_M = 25$ μM) and cGMP ($K_M = 237$ μM) and was resistant against the PDE inhibitor IBMX. The size of this protein was ~33 kDa as determined by gel filtration (Lavan et al. 1989). These data indicate a striking similarity to the results reported earlier for multifunctional PDE (Helfman et al. 1981; Helfman and Kuo 1982b), which, however, was not recognized at this point by Lavan et al. (1989). Only in 1991, another research group isolated this activity using the same protocol as Lavan et al. (1989) and found that this enzyme hydrolyzes both 3′,5′- and 2′,3′-cAMP, -cGMP, and -cCMP (Worby et al. 1991). Therefore, this activity most likely represents the rat homolog of the multifunctional PDE previously isolated from pig liver by HELFMAN (Helfman et al. 1981; Helfman and Kuo 1982b).

Another independent report on multifunctional PDE shows that this enzyme also occurs in human tissues. An IBMX-resistant PDE activity, which degraded cAMP ($K_M = 23$ μM) and cCMP ($K_M = 75$ μM) and was inhibited by inorganic phosphate, was purified from human liver tissue (Mendel et al. 1997). Gel filtration analysis revealed a globular protein with a molecular weight of ~38 kDa (Mendel et al. 1997), suggesting that this enzyme is the human homolog of multifunctional PDE. Interestingly, this enzyme is responsible for the conversion of the prodrug cHPMPC (1-[((*S*)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine) to the antiviral drug cidofovir (HPMPC; 1-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine) (Mendel et al. 1997). Thus, the activity of human multifunctional PDE may also have pharmacological implications. Mostly, the occurrence of multifunctional PDE was reported for liver tissue. However, cHPMPC-hydrolyzing activity, which is probably caused by multifunctional PDE, was also detected in various human cell lines from other organs, e.g., renal proximal tubule epithelial cells (RPTEC), normal human bronchial epithelial cells (NHBEC), or normal human epidermal keratinocytes (NHEK) (Mendel et al. 1997). This suggests that multifunctional PDE may be a ubiquitous enzyme that is important for cCMP metabolism in several tissues.

Taken together, the data suggest that multifunctional PDE is distinct from the cCMP PDE discussed above. Moreover, it is different from 2′,3′-cNMP-hydrolyzing PDE in brain and from ribonuclease, both of which are incapable of hydrolyzing 3′,5′-cNMPs (Helfman and Kuo 1982a). It also cannot be fully excluded that the low molecular weight protein identified as multifunctional PDE was formed by proteolytic degradation of a larger enzyme. It may as well be possible that multifunctional PDE can form oligomers with altered properties. This was, e.g., described in the past for PDE4 (Richter and Conti 2004).

2.1.3 PDE7A: A "Conventional" PDE That Hydrolyzes cCMP

While the molecular identity of "cCMP-specific" and "multifunctional" PDE is still unknown, a recent analysis of recombinant phosphodiesterases revealed significant cCMP-hydrolyzing activity of PDE7A. A detailed characterization of a recombinant PDE7A1/2 consensus sequence ("PDE7A1/2") revealed a cCMP K_M value of 135 μM, which is very similar to the corresponding K_M of "multifunctional" PDE (cf. Sect. 2.1.2). Moreover, PDE7A1/2 hydrolyzes cCMP more than 6-fold faster

than cAMP, which also resembles the properties of multifunctional PDE. Thus, PDE7A1/2 is a low-affinity and high-velocity enzyme for cCMP. However, unlike multifunctional PDE, PDE7A isoforms show a very high affinity for cAMP in the range of 100–200 nM (Bender and Beavo 2006) and a molecular weight of 57 kDa (PDE7A1) and 50 kDa (PDE7A2 and A3) (Michaeli 2006). Moreover, in contrast to multifunctional PDE, PDE7A is inhibited by IBMX (Wang et al. 2005), and experiments with PDE7A1 revealed that it cannot hydrolyze 2′,3′-cNMPs (Monzel et al. 2014). Thus, PDE7A isoforms represent a third type of cCMP-hydrolyzing activity, in addition to "cCMP-specific" and "multifunctional" PDE.

PDE7A1 is a cytosolic PDE, which is ubiquitously expressed. PDE7A1 in immune cells, specifically in T lymphocytes, has gained attention as a potential target for the treatment of inflammation (Bender and Beavo 2006; Francis et al. 2011). By contrast, PDE7A2 is mainly membrane-associated and its expression is limited to specific tissues, e.g., the heart (Bender and Beavo 2006). PDE7A-mediated cCMP hydrolysis may become important, when cells are infected by bacteria expressing nucleotidylyl cyclases, e.g., ExoY (*Pseudomonas aeruginosa*) (Monzel et al. 2014). ExoY produces large amounts of cyclic pyrimidine nucleotides, including cCMP, which may have toxic effects (Beckert et al. 2014; Chapter 4 by K.A. Morrow et al., "The *Pseudomonas aeruginosa* exoenzyme Y: A promiscuous nucleotidyl cyclase edema factor and virulence determinant"). Under such conditions, a low-affinity and high-velocity PDE for cCMP like PDE7A would be ideally suited to detoxify the cell (Monzel et al. 2014). More studies are needed to show whether there is a (patho)physiological role of the PDE7A1/2-cCMP system.

Interestingly, PDE7A was the only one out of thirteen studied PDEs (Reinecke et al. 2011, 2013; Monzel et al. 2014) that hydrolyzed significant amounts of cCMP. This indicates that cCMP hydrolysis is rather a task of "uncommon" enzymatic entities like "cCMP-specific" or "multifunctional" PDEs (see preceding sections), but an exception among the established class I phosphodiesterases.

2.1.4 cCMP-Degrading and Multifunctional PDEs in Bacteria, Algae, and Plants

PDEs and non-canonical cyclic nucleotides are also present in higher plants (Chapter 5 by C. Marondedze et al., "Cyclic nucleotide monophosphates in plants and plant signaling") and lower organisms like algae and bacteria. However, research on plant PDEs is surprisingly underrepresented as compared to research on the mammalian enzymes. Moreover, similar to the mammalian PDEs, the role of non-canonical cNMPs as substrates of plant PDEs was investigated mainly in the 1970s and 1980s, but the research community seems to have lost interest in the past 20 years. In the following, the most important publications about cCMP-degrading and multifunctional PDEs in bacteria, algae, and higher plants are discussed.

Cellular extracts from the bacterium *Serratia marcescens* contain a PDE that not only prefers cAMP ($K_M = 0.52$ mM), but also hydrolyzes cCMP (Okabayashi and Ide 1970). However, at a substrate concentration of 5 mM, cCMP hydrolysis occurred at only 17% of the cAMP hydrolysis rate. Moreover, the enzyme exhibited an extremely high K_M value of 20 mM (Okabayashi and Ide 1970). Thus, cCMP

hydrolysis by this enzyme is probably without relevance. Nevertheless, it should be noted that this enzyme is activated by Fe^{2+} (doubling of cAMP hydrolysis rate at 1 mM of Fe^{2+}) (Okabayashi and Ide 1970), which reminds of the cCMP-specific PDE isolated from rat liver (Kuo et al. 1978). Another non-mammalian cCMP PDE was isolated from the green algae *Chlamydomonas reinhardtii* (Fischer and Amrhein 1974). The enzyme showed a pH-optimum at 8.5, which is remarkable, because phosphodiesterases from higher plants mostly have acidic pH optima (Fischer and Amrhein 1974). The K_M value for cAMP was 85 μM and maximum activity was obtained in the presence of a combination of cysteine and Mg^{2+}. Moreover, the enzyme was activated by Mn^{2+} (Fischer and Amrhein 1974). Interestingly, at a substrate concentration of 2 mM, the enzyme hydrolyzed cCMP at 3-fold the rate of cAMP degradation (Fischer and Amrhein 1974).

A crude homogenate of *Phaseolus vulgaris* was fractionated by $(NH_4)_2SO_4$ precipitation. Gel filtration of the 37–48% fraction yielded two enzymatically active peaks with very distinct apparent molecular masses (34 and 7.6 kDa) (Brown et al. 1977). The enzymatic activity of the 7.6 kDa peak is very surprising, because the active agent is rather a peptide than a conventional PDE protein. Both peaks exhibited an activity optimum at pH 6.5 and hydrolyzed 3′,5′-cAMP, cGMP, cUMP, cIMP, cXMP, cCMP, and cdTMP at similar hydrolysis rates (Brown et al. 1977). Unlike the mammalian multifunctional PDE (see Sect. 2.1.2), the *Phaseolus* PDE did not hydrolyze 2′,3′-cAMP (Brown et al. 1977). It was suggested that the two peaks are either caused by aggregation of the enzyme or represent protein complexes that contain the same PDE enzyme (Brown et al. 1977).

A kind of multifunctional cCMP-hydrolyzing PDE with a temperature optimum of 40°C was isolated from nine- to ten-day pea seedlings (*Pisum sativum*) by $(NH_4)_2SO_4$ fractionation, pH fractionation, and gel filtration (Lin and Varner 1972). The enzyme hydrolyzes both 3′,5′-cNMPs and 2′,3′-cNMPs and was slightly stimulated by Mn^{2+}, Co^{2+}, and Zn^{2+}. Interestingly, unlike mammalian PDEs that produce exclusively 5′-NMPs from 3′,5-cNMPs, the pea seedling enzyme preferentially yielded 3′-AMP when hydrolyzing 3′,5′-cAMP (3′-AMP:5′-AMP ratio ~7:1). NaF selectively inhibited 3′,5′-cAMP hydrolysis, but did not affect 2′3′-cAMP degradation. Michaelis–Menten analysis revealed relatively low substrate affinities. The K_M values for 3′,5′-cAMP, 3′,5′-cCMP, and 3′,5′-cUMP were 0.9 mM, 1.81 mM, and 0.58 mM, respectively (Lin and Varner 1972). Like the mammalian multifunctional PDE, the pea seedling enzyme was inhibited by phosphate, but resistant to theophylline. However, unlike the mammalian enzyme, the pea seedling PDE showed a relatively large molecular mass of 350 kDa and a more acidic pH optimum at pH 5.4–6.0 (Lin and Varner 1972). The enzyme showed a higher hydrolysis rate for 2′,3′-cAMP as compared to 3′,5′-cAMP. Lin and Varner (1972) have suggested that the enzyme may be important for the hydrolysis of 2′,3′-cNMPs that are formed in plants during mRNA degradation.

A distinct type of cCMP-hydrolyzing pea seedling PDE was isolated from three-day pea seedling roots. After $(NH_4)_2SO_4$ precipitation, two species of cCMP PDEs were detected (Chiatante et al. 1990). The first one was present in the 50–75% fraction and showed features of a multifunctional PDE, degrading cAMP and

cGMP as well as cCMP. The activity was further purified by gel filtration chromatography (Chiatante et al. 1990). The second kind of cCMP-degrading activity was found in the 75–100% fraction and showed no activity for cGMP, but hydrolyzed cAMP and cCMP with high specificity for the latter (Chiatante et al. 1990). This enzyme was purified by affinity chromatography and subjected to Michaelis–Menten kinetics, revealing a cCMP K_M value of ~0.5 mM and a V_{max} of 122 nmol/min/mg (Chiatante et al. 1990). The pea root cCMP PDE was activated by Fe^{3+} and by Mg^{2+} and Ca^{2+}, while activity was eliminated in the presence of EDTA (Chiatante et al. 1990). Unlike the rat liver cCMP PDE (Kuo et al. 1978) and the multifunctional pea seedling PDE (Lin and Varner 1972), the pea root cCMP PDE was sensitive to theophylline, which caused an 88% inhibition at a concentration of 0.5 mM (Chiatante et al. 1990). Interestingly, theophylline also inhibited the elongation of pea roots (Chiatante et al. 1990). However, it was not finally proven that this effect resulted from PDE inhibition.

cCMP-hydrolyzing PDEs were not only isolated from legumes like peas and beans, but also from the cotyledons of garden lettuce (*Lactuca sativa*). Sequential application of $(NH_4)_2SO_4$ precipitation, chromatographic methods, gel electrophoresis (Chiatante et al. 1986), or isoelectric focusing (Chiatante et al. 1987) resulted in an enzyme with a molecular mass of ~62 kDa, which hydrolyzed 3′,5′- as well as 2′,3′-cNMPs. The K_M values ranged from 0.67 mM (3′,5′-cCMP) to 1.12 mM (2′,3′-cAMP) (Chiatante et al. 1987). Thus, this enzyme represents another plant multifunctional PDE with a similar substrate profile and affinity as the previously reported mammalian multifunctional PDE (Helfman et al. 1981; Helfman and Kuo 1982a). Moreover, similar to several other cCMP-degrading PDEs described in the literature (Kuo et al. 1978; Okabayashi and Ide 1970; Chiatante et al. 1990), the *Lactuca* enzyme was stimulated by Fe^{2+} (and Fe^{3+}) with the highest relative stimulation observed for cGMP degradation (Chiatante et al. 1986). Interestingly, the enzyme exhibited a relatively low pH optimum at pH 5.5 and an intriguingly high temperature optimum of 60°C with a dramatical decline in activity at higher temperatures (Chiatante et al. 1987). Moreover, this enzyme seems to have more than one binding site for cNMPs (Chiatante et al. 1987). Later, also a highly purified immunoaffinity preparation of the *Lactuca* multifunctional PDE was reported (Chiatante et al. 1988).

Potato tubers also contain a multifunctional PDE, which was purified to homogeneity (Zan-Kowalczewska et al. 1984). The enzyme has a molecular mass between 79 and 81 kDa (dependent on measurement method), is able to form oligomers, and hydrolyzes 2′,3′-cAMP with a K_M value of 400 μM (Zan-Kowalczewska et al. 1984). When the velocity of hydrolysis in the presence of 5 mM of substrate is set to 100% for 2′,3′-cAMP, the other cNMPs are degraded at rates of 20% (2′,3′-cGMP), 44% (2′,3′-cCMP), 15% (3′,5′-cAMP), 12% (3′,5′-cGMP), and 15% (3′,5′-cCMP), making this enzyme a multifunctional PDE with preference for 2′,3′-cAMP (Zan-Kowalczewska et al. 1984). Moreover, Zan-Kowalczewska et al. (1984) revealed a contamination of the potato tuber PDE by a pyrophosphatase that exhibits an almost identical migration behavior in standard SDS-gel electrophoresis. Only when the gel was run without prior

reduction of the samples, it was possible to separate the two enzymes (Zan-Kowalczewska et al. 1984). Thus, potential contamination of plant PDEs by pyrophosphatase should be considered, specifically, when activity against the pyrophosphate linkage in NAD^+ is observed (Zan-Kowalczewska et al. 1984). A surprising property of the potato tuber multifunctional PDE is a selective loss of activity against 3′,5′-cNMPs during storage at pH 7.5 and 4°C, while hydrolysis of 2′,3′-cNMPs remains unaffected (Zan-Kowalczewska et al. 1987). Zan-Kowalczewska et al. (1987) hypothesize that such selective inactivation mechanisms may be responsible for some conflicting reports on the substrate specificities of higher plant cNMP PDEs.

2.2 cUMP-Hydrolyzing Enzymes

In contrast to the reports about cCMP-hydrolyzing activities, the data on cUMP-degrading enzymes are only scarce, but reach back at least five decades. A PDE activity was found in rabbit brain that preferentially hydrolyzes cAMP, but also accepts cUMP at ~11% and cGMP at 33% of the rate of cAMP hydrolysis (Drummond and Perrott-Yee 1961). A few years later, another publication reported on a similar activity detected in rat brain cortex preparation that hydrolyses cGMP at 70% and cUMP at 10% of the cAMP hydrolysis rate (Cheung 1967).

The later Nobel laureate SUTHERLAND mentioned an unpublished observation in a review article (Sutherland and Rall 1960), showing that a cardiac phosphodiesterase hydrolyzed cUMP and cIMP at velocities of ~60 and 100% of the cAMP hydrolysis rate, respectively. Later, Hardman and Sutherland (1965) reported on a cUMP-hydrolyzing enzyme from dog cardiac tissue, which was mainly associated with the particulate fraction and hydrolyzed cUMP with even higher speed than cAMP (Hardman and Sutherland 1965). This enzyme was mainly found in heart, while all other tissues contained less cUMP-degrading activity (Hardman and Sutherland 1965). Later on, this activity (173-fold purified, related to cAMP-hydrolysis) was characterized in more detail by Nair (1966). However, in contrast to the findings reported by Hardman and Sutherland (1965), the data of Nair (1966) show only a slow cUMP degradation rate of 12–15% of cAMP hydrolysis. This cannot be explained by insufficient saturation of the enzyme, since Nair (1966) used even higher substrate concentrations than Hardman and Sutherland (1965). It could be speculated that the lower cUMP hydrolysis rate reported by Nair (1966) may be due to the additional purification step with DEAE cellulose chromatography, which may have removed most of the cUMP degrading enzyme. In fact, Hardman and Sutherland (1965) had already suggested that their enzyme preparation may contain more than one PDE, because the PDE inhibitors theophylline and caffeine had different inhibitory potencies against cUMP or cAMP hydrolysis. The enzyme preparation isolated by Nair (1966) hydrolyzed also cGMP and cIMP at 33% and 55–65% of the cAMP hydrolysis rate, respectively. Interestingly, neither the enzyme from rabbit brain (Drummond and Perrott-Yee 1961) nor from dog heart (Nair 1966) degraded cCMP.

Another cUMP-degrading activity was found in the 100,000 g sediment of rat epididymal fat tissue (Klotz and Stock 1971). A characterization of enzyme kinetics revealed K_M values of ~100 μM and 580 μM for cAMP and cUMP, respectively. The V_{max} values were 5.7 nmol/mg/min (cAMP) and 11.4 nmol/mg/min (cUMP) (Klotz and Stock 1971). These results, however, should be considered with caution, since the methods used to follow the enzymatic reaction were not very sensitive (colorimetric detection of inorganic phosphate liberated from cAMP) (Klotz and Stock 1971). For the next 40 years, to the best of our knowledge, the paper by Klotz and Stock (1971) was the last report on cUMP-degrading activities.

Only in 2011, an analysis of eight different recombinant PDEs by HPLC-MS revealed that the PDEs 3A, 3B, and 9A exhibit pronounced cUMP hydrolysis (Reinecke et al. 2011). At a substrate concentration of 10 μM, PDE3A and 3B hydrolyzed cIMP at almost the same rate as cAMP, while cUMP was degraded at 31% (PDE3B) or 84% (PDE3A) of the cAMP hydrolysis rate (Reinecke et al. 2011). PDE9A showed cUMP and cIMP hydrolysis at a similar rate as cAMP degradation (Reinecke et al. 2011). The cUMP-hydrolytic activity of PDE3A was later confirmed with a fluorescence-based assay using MANT (N'-methylanthraniloyl)-labeled cUMP (Reinecke et al. 2013). Neither PDE3A nor PDE3B or PDE9A accept cCMP as a substrate.

These properties are very similar to the properties reported decades earlier for various cUMP-degrading activities.The cardiac cUMP PDE (Sutherland and Rall 1960; Hardman and Sutherland 1965; Nair 1966) may in fact represent PDE3A, which, as PDE3A1 isoform, is highly expressed in cardiac and vascular myocytes (Omori and Kotera 2007). The cUMP-degrading PDE activity discovered in rabbit brain (Drummond and Perrott-Yee 1961), however, may be due to PDE9A which is highly expressed not only in brain, but also in other tissues like spleen, kidney, small intestine, colon, prostate, and placenta (Omori and Kotera 2007). Finally, the cUMP hydrolysis in rat adipose tissue reported by Klotz and Stock (1971) may be caused by PDE3B, which is predominantly expressed in adipose tissue, but not in the heart (Omori and Kotera 2007). Thus, the systematic screening of recombinant PDEs with different substrates (Reinecke et al. 2011) led to candidates that may now help to unveil the identity of the previously described cUMP-degrading activities.

2.3 cIMP-, cXMP-, and cTMP-Hydrolyzing Enzymes

In contrast to the cyclic pyrimidine nucleotides cCMP and cUMP, the non-canonical cyclic purine nucleotides cIMP, cXMP, and cTMP were rather neglected in the second messenger-related research of the past five decades. Although detection of these cyclic nucleotides in rat tissue by fast atom bombardment mass spectrometry was reported by Newton and Salih (1986), intracellular occurrence of cIMP, cXMP, and cTMP could not be confirmed in later analyses (Beste and Seifert 2013; Beste et al. 2013). Only very recently, significant amounts of cIMP were identified in heart and testes of adult zebrafish (Dittmar et al. 2015).

There are only a few scattered reports on the physiological actions of cIMP, cXMP, and cTMP. For example, perfusion of rat livers with cIMP resulted in stimulation of gluconeogenesis with cIMP being as potent as cAMP (Conn et al. 1971). Moreover, only very recently, it was suggested that cIMP may be formed by soluble guanylyl cyclase (sGC) under hypoxic conditions and mediate contraction of coronary arteries (Chen et al. 2014; Gao et al. 2015; Chapter 11 by S.W.S. Leung et al., "3′,5′-cIMP as potential second messenger in the vascular wall"). This hypothesis is still controversially discussed (Seifert 2015a), but, if verified, it would implicate the existence of cIMP-hydrolyzing PDEs. An example of a biological effect of cTMP is the study of Brus et al. (1984) that demonstrated that intracerebroventricular administration of cTMP (as well as cCMP and cUMP) induced hypothermia and reduced motor activity and locomotor activity in rats. Moreover, specifically cTMP reduced thermal pain sensitivity in the hot-plate test (Brus et al. 1984). To the best of our knowledge, there is no data available on the physiological effects of cXMP. Moreover, a second messenger function of cXMP and cTMP is very unlikely (Seifert et al. 2015).

Since no systematic research has been performed on cIMP-, cXMP-, and cTMP-hydrolyzing PDEs, it is difficult to comprehensively summarize the literature on this topic. In most instances, these cNMPs were studied along with other cNMPs in experiments that addressed substrate selectivity of PDEs for cAMP, cGMP, cCMP, or cUMP.

Due to its structural similarity to the established second messenger cGMP (Fig. 1), it is conceivable that cIMP is accepted as a substrate by cGMP-hydrolyzing PDEs. In fact, a screen of several recombinant "conventional" PDEs at a substrate concentration of 10 μM revealed that the dual-specific PDEs 1B, 2A, 3A, and 3B as well as the cGMP-specific PDEs 5A and 9A all hydrolyze cIMP at comparable or even higher rates than cGMP (Reinecke et al. 2011). Even PDE4B, which is a cAMP-"selective" PDE, hydrolyzes 10 μM of cIMP at about 34% of its velocity for cAMP (Reinecke et al. 2011). Moreover, PDE1A3 hydrolyzed cIMP with a velocity comparable to cGMP at a substrate concentration of 3 μM (Monzel et al. 2014). The cIMP-hydrolyzing activity of PDE1B, 3A, 5A, and 9A was additionally confirmed by a fluorescence-based method using MANT-labeled cIMP as a substrate (Reinecke et al. 2013). At a concentration of 10 μM, cXMP was significantly (>45% of cGMP hydrolysis rate) hydrolyzed by the dual-specific PDEs 1B, 3A, 3B and the cGMP-specific PDEs 5A and 9A, while cTMP was mainly hydrolyzed by PDE3A, 5A and 9A (Reinecke et al. 2011). Moreover, experiments with crude homogenates from PDE-expressing Sf9 cells suggest that PDE1A3 hydrolyzes cXMP and PDE7A1 digests cTMP (Monzel et al. 2014).

As discussed in the preceding section, the cardiac cUMP-degrading enzyme described several decades ago also accepted cIMP (Sutherland and Rall 1960; Nair 1966). Thus, this enzyme may represent PDE3A, which is also expressed in cardiac tissue (Omori and Kotera 2007) and hydrolyzes cUMP and cIMP in addition to cAMP (Reinecke et al. 2011). Furthermore, mammalian tissues also contain a "multifunctional" PDE activity (Helfman and Kuo 1982a), which does not discriminate between 3′,5′- and 2′,3′-cNMPs and hydrolyzes the 3′,5′- as well

as the 2′,3′-esters of cIMP, cCMP, cUMP, cAMP, and cGMP (Newton et al. 1999).

Several reports describe cIMP-hydrolyzing PDE activities in plants and bacteria. As mentioned in Sect. 2.1.4, a protein in *Phaseolus vulgaris* hydrolyzes not only cIMP, but also cAMP, cGMP, cUMP, cXMP, cCMP, and cTMP at comparable hydrolysis rates (Brown et al. 1977). In pea seedlings (*Pisum sativum*) a multifunctional PDE activity was isolated, which hydrolyzes both 3′,5′- and 2′,3′-cNMPs. At a substrate concentration of 4 mM, this enzyme digests 2′,3′-cUMP with highest velocity. Both 3′,5′-cIMP and 3′,5′-cAMP are hydrolyzed at a rate of 40–45% and 3′,5′-cTMP at a rate of 26% of the 2′,3′-cUMP degradation speed (Lin and Varner 1972). A cIMP-hydrolyzing PDE activity was also reported for the green algae *Chlamydomonas reinhardtii* (Fischer and Amrhein 1974). Related to the velocity of cAMP hydrolysis, which was set to 100%, this enzyme also hydrolyses cCMP (300%), cGMP (50%), cTMP (35%), cUMP (30%), and cIMP (45%) at a substrate concentration of 2 mM (Fischer and Amrhein 1974).

The social amoeba *Dictyostelium discoideum* expresses a 3′,5′-cGMP-specific PDE, which is activated 4-fold by low concentrations of cGMP. Despite its structural similarity to cGMP, however, cIMP is not binding to the activator site and cannot stimulate activity of this PDE (Van Haastert and Van Lookeren Campagne 1984). Nevertheless, cIMP is readily hydrolyzed at the catalytic site of the non-activated enzyme with a K_M value of 28 μM and a V_{max} of 1,200 pmol/min/mg. If the enzyme is activated in presence of the hydrolysis-resistant cGMP derivative 8-bromoguanosine-3',5'-monophosphate, cIMP is hydrolyzed with the same V_{max} as in case of the non-activated enzyme, but with higher affinity ($K_M = 6.5$ μM) (Van Haastert and Van Lookeren Campagne 1984). Furthermore, the bacterial PDE from *Serratia marcescens* reported by Okabayashi and Ide (1970) hydrolyses cIMP at ~70% and cTMP at ~54% of the rate for cAMP. It should be noted, however, that for these assays an extremely high substrate concentration of 5 mM was chosen (Okabayashi and Ide 1970). While the K_M value of cAMP at this enzyme was 520 μM, the affinity of cIMP and cTMP was not determined by Okabayashi and Ide (1970).

There are also publications that suggest an inhibitory effect of cIMP on PDEs. It should be noted that inhibition of PDEs by cIMP does not exclude that cIMP is also hydrolyzed by the corresponding enzyme. This, however, was not investigated in the publications discussed in the following. For example, a partially purified PDE from human lung tissue, which hydrolyzed both cAMP and cGMP with a K_M value of 0.4 μM, was inhibited by 50% in the presence of a cIMP concentration of only 2 μM, while cTMP, cCMP, and cUMP were ineffective (Bergstrand et al. 1978). Moreover, in the presence of 1 μM of cCMP, the activity of the cCMP-specific PDE described by Kuo et al. (1978) was reduced to ~50% by 1 mM of cIMP. It should be noted, however, that this effect may be physiologically irrelevant, because it is unlikely that cIMP concentrations as high as 1 mM are reached in living organisms.

An inhibitory effect of cIMP (and cGMP) was also found in crude enzyme preparations from cat heart or rat brain that were prepared by ammonium sulfate precipitation (Harris et al. 1973). Cat heart PDE-mediated degradation of 0.06 μM

of cAMP was inhibited by cIMP and cGMP with IC_{50} values of 2 μM and 1 μM, respectively. All other examined 3′,5′-cyclic nucleotides were either inactive (cCMP) or had IC_{50} values of >1,500 μM (cUMP, cTMP) (Harris et al. 1973). The rat brain PDE, which was studied under the same conditions, was less sensitive with IC_{50} values of 700 μM and 940 μM for cGMP and cIMP, respectively, while cUMP, cCMP, and cTMP were ineffective (Harris et al. 1973).

Another example of an inhibitory cIMP effect was reported for a cAMP-hydrolyzing enzyme enriched from a crude extract of dormant tubers from Jerusalem artichoke (*Helianthus tuberosus*) by sequential application of centrifugation, dialysis, ammonium sulfate precipitation and gel chromatography (Giannattasio et al. 1974). This enzyme hydrolyzed cAMP with a K_M value of 680 μM, showing a pH optimum between 5.2 and 5.4. At a substrate concentration of 100 μM, cAMP hydrolysis was strongly inhibited by inorganic phosphate or pyrophosphate. Moreover, cAMP hydrolysis was inhibited by 52, 65, and 38% in the presence of 1 mM of cIMP, cUMP, and cCMP, respectively (Giannattasio et al. 1974). Furthermore, three cAMP-hydrolyzing PDE activities were detected in crude preparations from *Dictyostelium discoideum* (Yamasaki and Hayashi 1982) and were localized intracellularly ("soluble"), in the membrane ("particular") and in the incubation medium ("extracellular"). All activities were inhibited by cIMP with K_i values ≤100 μM (Yamasaki and Hayashi 1982).

2.4 How Reliable Are the Historical Data on PDE-Mediated cNMP Hydrolysis?

The numerous publications discussed so far report on several enzymatic activities for cCMP, cUMP, cIMP, cXMP and cTMP with kinetic parameters (K_M and V_{max}) that are often very untypical, when compared to "conventional" class I PDEs. Many of these publications were from the time between 1960 and 1990 and very often, the molecular identity of these PDE activities was not determined. Moreover, analytical methods were not so highly developed as nowadays, which may also have led to erroneous results. Thus, in this section, the two most common methods to determine PDE activity will be discussed and the reliability of the results obtained by these techniques will be critically evaluated.

Most of the older publications use essentially two methods for the characterization of PDEs. The first method, which was modified in many ways, is derived from a two-step assay developed by Thompson and Appleman (1971). The enzyme preparation is incubated with radioactively labeled (tritiated) substrate and the reaction is stopped by heating the sample. To separate educts and products of the PDE reaction, a 5′-nucleotidase (e.g., snake venoms from *Crotalus atrox, Crotalus adamanteus*, or *Ophiophagus hannah*) is used to quantitatively convert the NMPs to the corresponding nucleosides, while the non-hydrolyzed cNMPs remain unaffected. After inactivation of the 5′-nucleotidase, the nucleosides are separated from the cNMPs by using an anion exchange resin, which selectively retains cNMPs, while nucleosides appear in the eluate and are quantified by scintillation

counting. This method or modifications of it (e.g., a one-step assay with direct addition of the nucleotidase to the PDE/substrate mixture) was used in almost all of the papers reporting on low-affinity cCMP PDE and multifunctional PDE (Cheng and Bloch 1978; Kuo et al. 1978; Helfman et al. 1978, 1981; Shoji et al. 1978a, b; Wei and Hickie 1983; Newton and Salih 1986; Helfman and Kuo 1982a, b; Lavan et al. 1989). Moreover, this technique can be found in several publications about cCMP-degrading plant PDEs (Chiatante et al. 1986, 1987, 1988, 1990; Zan-Kowalczewska et al. 1987) and in some of the papers discussing PDE-inhibitory effects of cIMP (Bergstrand et al. 1978; Harris et al. 1973).

The second and older method was published by Butcher and Sutherland (1962) and performed similar to the method described by Thompson and Appleman (1971), except that nucleosides and cNMPs were not separated after incubation with the 5′-nucleotidase/snake venom. Instead, the released phosphate was quantified by a colorimetric reaction. This method and modifications of it that also determine phosphate were used in several papers describing cUMP-hydrolyzing activities (Hardman and Sutherland 1965; Nair 1966; Cheung 1967; Klotz and Stock 1971) as well as in publications about bacterial and plant PDEs (Okabayashi and Ide 1970; Fischer and Amrhein 1974; Brown et al. 1977).

A major problem of both methods is that they do not provide any detailed information about the chemical identity of the reaction products. In order to achieve that, chromatographic methods were used, e.g., paper chromatography or thin layer chromatography. However, these methods also give only very superficial and indirect information. A problem of the radiometric method is separation of the radiolabeled nucleosides from the non-hydrolyzed cNMPs by anion exchange resins. This, however, bears the risk of technical errors, e.g., of overloading the column, leading to the elution of educt, which may be mistakenly interpreted as product. A major problem of the second method, which makes use of colorimetric phosphate detection, is low sensitivity. This may be the reason, why in many papers cNMP concentrations of several hundred μM or even in the low mM-range were added to the reaction mixtures. One might speculate that the detection of low-affinity PDEs was possibly favored by the high cNMP concentrations required by such analytical methods. The problem of the unphysiologically high cNMP concentrations in the PDE assays was already recognized by Butcher and Sutherland (1962). This was addressed by using an indirect biological assay determining the effects of low cAMP concentrations on liver homogenates, which had been described earlier in one of the seminal papers of cAMP research (Rall and Sutherland 1958).

Compared to the two methods discussed above, the use of mass-spectrometry-based product identification was a big step forward. For example, the comparison of multifunctional PDE and cCMP PDE by Newton et al. (1999) was performed by fast atom bombardment mass spectrometry. However, although the specificity of this method may be sufficient for the detection of reaction products in enzyme digestion assays with defined sample composition, it is probably not accurate enough to unequivocally identify nucleotides in biological matrices (Beste and Seifert 2013). This is only possible with HPLC-MS/MS-based methods that became available during the past years (Seifert et al. 2015).

Moreover there are two other problems not directly associated with the assays and the analytical equipment. First, many experiments discussed in this chapter were performed during times, when methods of protein analytics were not as highly developed as nowadays. Furthermore, the expression of recombinant proteins in host organisms was not possible yet. Thus, it was very difficult to identify the proteins responsible for the described PDE activities and it cannot be excluded that some data were collected with enzyme mixtures rather than pure PDEs. This, however, makes interpretation of K_M and V_{max} values difficult. Second, data analysis was mostly performed by manual methods (e.g., Lineweaver–Burk plot), which did not reach the accuracy of modern computer-based non-linear regression.

Taken together, the decades-old reports on PDE activities for non-canonical PDEs should be regarded with caution, but certainly not dismissed. With the highly selective and specific analytical methods like HPLC-MS/MS, which became available during the past years (Seifert et al. 2015), it is now possible to re-address these old studies and to revive the research on low-affinity PDEs for non-canonical cyclic nucleotides. Moreover, cNMP specificity of the established PDEs can be determined more accurately than ever before (Reinecke et al. 2011; Monzel et al. 2014). The current knowledge about PDE-mediated hydrolysis of the non-canonical cNMPs, cCMP, and cUMP is summarized in Fig. 3.

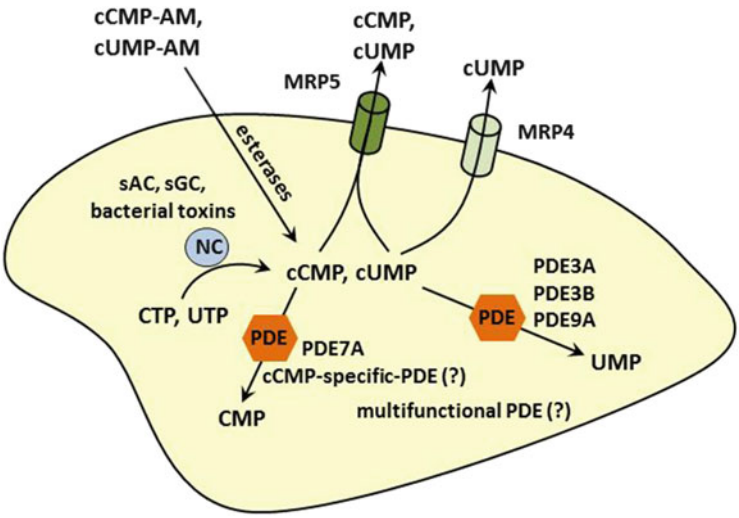

Fig. 3 Inactivation of the non-canonical cNMPs cCMP and cUMP by PDEs and MRPs. cCMP and cUMP are formed by nucleotidylyl cyclases (NC) like sAC, sGC, or some bacterial toxins. Experimentally, increase of intracellular cCMP and cUMP can be induced by the corresponding membrane permeable acetoxymethyl (AM)-esters, followed by esterase-mediated release of the cNMPs. Enzymatic degradation of cCMP is achieved by PDE7A, by a cCMP-specific PDE, or by multifunctional PDE. The PDEs 3A, 3B, and 9A hydrolyze cUMP. Moreover, cUMP may be degraded by a multifunctional PDE. Alternatively, cCMP can be exported by MRP5 in a low-affinity high-capacity transport. Similarly, cUMP is extruded by both MRP4 and 5. As indicated by the *question marks*, the molecular identities of the cCMP-specific PDE and the multifunctional PDE are still unknown

3 Inactivation of cNMPs by Outward Transport

In addition to enzymatic degradation by PDEs, which was extensively discussed in this chapter, active export is another important mechanism for the inactivation and disposal of cyclic nucleotides. Due to the focus of this chapter on PDEs, however, we will only discuss a limited number of representative publications describing MRP (multidrug resistance associated protein)-mediated transport of cNMPs in mammalian cells and tissues.

MRPs represent one of the seven transporter subfamilies (subfamily C) that form the superfamily of ATP binding cassette (ABC) transporters (Dallas et al. 2006). ATP hydrolysis provides the energy for MRP-mediated transmembraneous transport of various substrates (Dallas et al. 2006). MRP substrates show high structural diversity, including not only numerous cancer chemotherapeutics (e.g., daunorubicin, methotrexate, cisplatin, or fluoropyrimidines), estradiol derivatives, or glutathione conjugates (Dallas et al. 2006), but also antiviral drugs, antibiotics, and various cardiovascular drugs (Wen et al. 2015). Among the nine MRP transporters, specifically MRP4 and MRP5 (Dallas et al. 2006; Sager 2004) and MRP8 (Guo et al. 2003; Sager 2004) accept cyclic nucleotides as substrates.

MRP4 is mainly expressed in brain, prostate, lungs, kidney, testes, ovary, and pancreas (Dallas et al. 2006). Characterization of MRP4 substrate selectivity with membrane vesicles isolated from baculovirus-infected insect cells revealed that MRP4 transports both cGMP ($K_M = 9.7$ μM, $V_{max} = 2.0$ pmol/mg/min) and cAMP ($K_M = 44.5$ μM, $V_{max} = 4.1$ pmol/mg/min) (Chen et al. 2001). MRP5 is expressed ubiquitously with high levels in brain, skeletal muscle and heart (Dallas et al. 2006). MRP5-mediated transport of cAMP and cGMP was characterized in great detail in MRP5-transfected V79 hamster lung fibroblasts, yielding a cGMP K_M value of 2.1 μM and a much lower affinity of 379 μM for cAMP (Jedlitschky et al. 2000). MRP8 was identified in breast, testes, liver, and placenta (Dallas et al. 2006). Guo et al. (2003) demonstrated that MRP8 expression was associated with enhanced extrusion of both cAMP and cGMP. Moreover, molecular modelling and molecular dynamics simulations revealed putative binding sites of MRP8 for cGMP and the cancer chemotherapeutic drug 5-fluoro-2′-deoxyuridine-5′-monophosphate (Honorat et al. 2013).

Although cyclic nucleotide transport by MRPs 4 and 5 and its physiological relevance were discussed controversially (Borst et al. 2007), the data accumulated during the past years support the importance of MRP-mediated cNMP extrusion. For example, MRP4 was upregulated during proliferation of arterial smooth muscle cells and inhibition of MRP4 increased intracellular cAMP and cGMP concentrations (Sassi et al. 2008). Blocking MRP4 function had an antiproliferative effect on smooth muscle cells, presumably due to increased cAMP-mediated activation of the PKA/CREB pathway (Sassi et al. 2008). The role of MRP4 for cAMP and cGMP homeostasis might become important for future therapies of cardiovascular diseases. For example, MRP4 is upregulated in pulmonary arteries not only from mice exposed to hypoxia, but also from patients with idiopathic pulmonary arterial hypertension (Hara et al. 2011). Moreover, MRP4-

deficient mice were resistant to hypoxic pulmonary hypertension and the disease was reversed by treatment with the MRP4 inhibitor MK571 (Hara et al. 2011). MRP4 is also the main MRP isoform expressed on cardiomyocytes. Adult (9 months old) MRP4-deficient mice show increased isoproterenol-induced cardiomyocyte cAMP generation, enhanced contractility and cardiac hypertrophy (Sassi et al. 2012). Interestingly, unlike the 9-month-old *Mrp4*$^{-/-}$ mice, the younger 3-month-old animals were able to compensate the lack of MRP4 by upregulation of PDE expression (Sassi et al. 2012), suggesting that the importance of MRP-mediated cNMP extrusion is age-dependent. MRP4 is also highly important for the regulation of intracellular cAMP concentrations in human leukemia cells. As reported by Copsel et al. (2011), blockade of MRPs by probenecid strongly intensified the anti-proliferative effect of intracellular cAMP generated after H_2R stimulation by amthamine in U937 promonocytes. Moreover, in combination with the PDE4 inhibitor rolipram, probenecid enhanced differentiation of U937 cells to monocyte-like cells (Copsel et al. 2011). The probenecid-mediated enhancement of the anti-proliferative and differentiation-inducing effect of amthamine was mimicked by MRP4 knockdown with MRP4-shRNA (Copsel et al. 2011). The principle of probenecid-mediated enhancement of cAMP responses was also used to intensify and unmask the effect of various H_2R ligands on U937 cell differentiation and the accompanying upregulation of formyl peptide receptor (FPR) (Werner et al. 2015).

It has even been demonstrated very recently that MRP4-mediated cGMP export from smooth muscle cells is of comparable importance as hydrolysis by PDE5, and is essential for the regulation of cGMP-mediated vascular smooth muscle relaxation (Krawutschke et al. 2015; Stangherlin and Zoccarato 2015). Interestingly, competition of cAMP and cGMP for MRP4 also mediates a cAMP/cGMP crosstalk. Induction of a cAMP signal by isoproterenol via the β_2AR causes an elevation of intracellular cGMP concentration, which is caused by cAMP-mediated inhibition of cGMP export (Krawutschke et al. 2015; Stangherlin and Zoccarato 2015).

A similar mechanism as reported for MRP4 by Krawutschke et al. (2015) was already suggested more than a decade earlier for MRP5 (Xu et al. 2004a, b). In this case, cGMP export by MRP5 modulated NO/cGMP-mediated relaxation of rat pial arterioles (Xu et al. 2004a). Moreover, intracellular cAMP inhibited MRP5-driven cGMP efflux in rat pial arterioles and stimulated PDE5-mediated cGMP hydrolysis at the same time (Xu et al. 2004b). The inhibition of cGMP extrusion by cAMP, however, seemed to be of higher importance than the cAMP effect on PDE5-mediated cGMP breakdown (Xu et al. 2004b). A role of cGMP export by MRP5 is also suggested by more recent findings that show an influence of both MRP5 and PDE5 expression on the tonic phenotype of smooth muscle cells from fundus and antrum (Al-Shboul et al. 2013).

These examples illustrate the physiological importance of MRP-mediated cAMP and cGMP export. Due to the limitations of this chapter, however, we cannot comprehensively discuss the literature about cNMP extrusion by MRPs. While there are many reports on the export of cAMP and cGMP, the ability of MRPs to transport non-canonical cNMPs like cUMP, cCMP, and cIMP was much less

investigated. There is only one recent publication, which shows that cCMP is exported by MRP5 and this transport is not saturated at cCMP concentrations of 2.5 mM (Laue et al. 2014, Fig. 3). Thus, similar to some of the cCMP PDEs discussed above, MRP5 represents a low-affinity and high-capacity mechanism for the export of cCMP. Moreover, cUMP is accepted as a substrate by both MRP4 and MRP5 (Laue et al. 2014, Fig. 3). MRPs 1, 2, 3, and 8, however, do not transport cCMP or cUMP (Laue et al. 2014). It is doubtful that MRP-mediated extrusion of cyclic pyrimidine nucleotide is important under homeostatic conditions, since intracellular concentrations of these cNMPs do not reach the millimolar range. However, MRPs may help to detoxify cells with pathologically high cCMP or cUMP concentrations, e.g., after infection with ExoY-positive *P. aeruginosa* bacteria. The exotoxin ExoY is a nucleotidylyl cyclase, which causes a dramatic increase in cUMP concentration in infected tissues (Bähre et al. 2015; Morrow et al. 2015). To the best of our knowledge, at the moment nothing is known about cIMP transport by MRPs.

Finally, it should be noted that MRP-mediated export of cyclic nucleotides is by no way a functional "dead end street." There is increasing evidence that extracellular cNMPs exert effects, e.g., via degradation products formed by enzymes on the cell surface (Chapter 12 by E.K. Jackson, "The discovery of 2′,3′-cNMPs in mammalian systems: From rodents to humans"). For example, after its extrusion from the cell, cAMP is degraded to adenosine, which can act at the corresponding receptors. The so-called cAMP-adenosine pathway was investigated in much detail with regard to renal physiology (Jackson and Raghvendra 2004), but seems to be of general importance for the regulation of the cellular response resulting from activation of Gαs-coupled GPCRs (Godinho et al. 2015). For example, exogenous cAMP reduces intracellular cAMP formation in cardiomyocytes and prevents myocardial hypertrophy and fibrosis in a mouse model of cardiac pressure overload (Sassi et al. 2014). This cAMP-mediated cardioprotection requires adenosine receptors, indicating that the extracellular cAMP-adenosine pathway is responsible for this effect (Sassi et al. 2014).

A similar pathway may exist for cGMP. For example, extracellular cGMP and its degradation products GMP and guanosine protect HT22 neuronal cells from oxidative glutamate toxicity (Albrecht et al. 2013). Moreover, astrocytic glutamate uptake is stimulated by guanosine, which can be enzymatically generated from GMP by ecto-5′-nucleotidase (Frizzo et al. 2003). Similarly, the amnesic effect of GMP in mice (Saute et al. 2006) and the anticonvulsant action of GMP in rats (Soares et al. 2004) require the formation of guanosine by an ecto-5′-nucleotidase. Extracellular effects may also exist for non-canonical cNMPs and their degradation products. For example, it has been reported more than two decades ago that the potential cUMP degradation products uridine and uracil nucleotides regulate cellular functions (Seifert and Schultz 1989). Furthermore, the cIMP/IMP degradation product inosine is a partial agonist at adenosine A_1 and A_3 receptors (Fredholm 2010).

4 Summary and Conclusions

Various literature reports of the past five decades suggest the existence of enzymes degrading non-canonical cNMPs, specifically cCMP and cUMP. However, as discussed in Sect. 2.4, many of these results have to be interpreted with caution, since specificity and sensitivity of the used analytical methods were often insufficient. Only with the advent of HPLC-MS/MS-based methods for detection and quantitation of cNMPs, a systematic analysis of occurrence and metabolism of non-canonical cNMPs became feasible (Seifert et al. 2015). Moreover, many of the studies discussed in this chapter were performed at times, when it was not possible yet to characterize the substrate profiles of highly purified recombinant PDEs. Thus, future studies will have to demonstrate whether the non-canonical cNMP-hydrolyzing activities described in the past can be assigned to conventional PDEs. A first step towards this goal was taken in recent publications (Reinecke et al. 2011; Monzel et al. 2014).

The physiological importance of PDE-mediated hydrolysis of non-canonical cNMPs is largely elusive. It is currently discussed that cCMP and cUMP (Seifert 2015b) as well as cIMP (Chen et al. 2014; Gao et al. 2015) may represent potential second messengers. Alternatively, cNMPs produced by bacterial toxins like ExoY may be important under pathophysiological conditions (Bähre et al. 2015). The cCMP- and cUMP-degrading PDEs could be responsible for detoxification of high cCMP- or cUMP concentrations. In fact, toxicity of cCMP has been recently demonstrated in S49 cells, where the membrane-permeable acetoxymethyl ester of cCMP, cCMP-AM, induced apoptosis (Wolter et al. 2015). The "multifunctional" PDEs that hydrolyze 3′,5′- as well as 2′,3′-cNMPs may also have a detoxifying function. The biological effects of 2′,3′-cNMPs and their downstream metabolites, however, are only partially elucidated and remain in the focus of future research (Chapter 12 by E.K. Jackson, "The discovery of 2′,3′-cNMPs in mammalian systems: From rodents to humans").

When the physiological role of PDE-mediated hydrolysis of canonical and non-canonical cNMPs is explored, the function of MRP proteins in regulating intracellular cNMP concentrations should be kept in mind. As discussed in Sect. 3, specifically MRP4 and 5 may be at least as important for intracellular cNMP homeostasis as PDEs. Interestingly, among the "established" PDEs, hydrolysis of cCMP is a very rare feature, which supports the notion that other mechanisms like MRP-mediated export are also important for cCMP disposal. Out of 13 studied enzymes only PDE7A showed considerable cCMP-hydrolyzing activity (Reinecke et al. 2011; Monzel et al. 2014).

The features of the cCMP-hydrolyzing enzymatic entities described in the literature rather do not fit into the concept of "conventional" class I PDEs. The low molecular mass, the high K_M values for cCMP, the stimulating effect of Fe^{2+}, and the resistance to well-established unselective PDE inhibitors like IBMX suggest that these activities are produced by a totally different class of enzymes. It remains to be elucidated, if the observed cCMP-hydrolyzing activities are physiologically relevant.

Taken together, enzymes that degrade non-canonical cyclic pyrimidine nucleotides represent an exciting new field of PDE research. During the past

decades research in this area was mainly hampered by the lack of suitable detection methods. With the advent of highly sensitive and highly specific HPLC-MS/MS-methods (Bähre et al 2015; Chapter 15 by V. Kaever and H. Bähre, "Mass-spectrometric analysis of non-canonical cyclic nucleotides"), however, the initially rather sluggish research on cCMP, cUMP, and cIMP was markedly stimulated, opening an entirely new field of second messenger research. Table 5 demonstrates impressively for the "conventional" PDEs that most of the enzyme kinetic parameters (K_M and V_{max}) for the non-canonical cNMPs have not been determined yet. cIMP, cXMP, and cTMP are not shown in Table 5, because, like for cCMP and cUMP, practically nothing is known, except that they are not hydrolyzed by PDE8A (Reinecke et al. 2011). Moreover, cTMP is resistant against PDEs 1A (Monzel et al. 2014) and 1B, 2A as well as 4B (Reinecke et al. 2011). cXMP is not hydrolyzed by PDE7A1 (Monzel et al. 2014).

Most surprisingly, Table 5 even reveals numerous gaps for K_M and V_{max} values of the well-established canonical cNMPs, cAMP, and cGMP. Thus, future research

Table 5 K_M and V_{max} values for cNMP-hydrolysis by "conventional" PDEs (splice variants are not listed separately)

	cAMP		cGMP		cCMP		cUMP	
	K_M (μM)	V_{max} (μmol/min/mg)	K_M (μM)	V_{max} (μmol/min/mg)	K_M (μM)	V_{max} (μmol/min/mg)	K_M (μM)	V_{max} (μmol/min/mg)
PDE1A	72.7–124	70–450	2.6–3.5	50–300	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]
PDE1B	10–24	10	1.2–5.9	30	n.s.[a]	n.s.[a]	---	---
PDE1C	0.3–1.1	---	0.6–2.2	---	---	---	---	---
PDE2A	30	120	10	123	n.s.[a]	n.s.[a]	n.s.[a]	n.s.[a]
PDE3A	0.18	3.0–6	0.02–0.15	0.34	n.s.[a]	n.s.[a]	---	---
PDE3B	0.38	8.5	0.28	2.0	n.s.[a]	n.s.[a]	---	---
PDE4A	2.9–10	0.58	---	---	---	---	---	---
PDE4B	1.5–4.7	0.13	---	---	n.s.[a]	n.s.[a]	n.s.[a]	n.s.[a]
PDE4C	1.7	0.31	---	---	---	---	---	---
PDE4D	1.2–5.9	0.03–1.56	---	---	---	---	---	---
PDE5A	290	1.0	2.9–6.2	1.3	n.s.[a]	n.s.[a]	---	---
PDE6A/B	700	---	15	2,300	---	---	n.s.[b]	n.s.[b]
PDE6C	610	---	17	1,400	---	---	---	---
PDE7A	0.1–0.2	0.11[b]	---	---	135[b]	0.75[b]	---	---
PDE7B	0.03–0.07	---	---	---	---	---	---	---
PDE8A	0.06	---	n.s.[a]	n.s.[a]	n.s.[a]	n.s.[a]	n.s.[a]	n.s.[a]
PDE8B	0.10	---	---	---	---	---	---	---
PDE9A	230	---	0.7–0.17	---	n.s.[a]	n.s.[a]	---	---
PDE10A	0.22–1.1	---	13–14	---	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]
PDE11A	2.0–3-2	---	0.95–2.1	---	n.s.[b]	n.s.[b]	n.s.[b]	n.s.[b]

cCMP and cUMP are listed as examples for non-canonical cyclic nucleotides. If not otherwise indicated, enzyme kinetic parameters are taken from Bender and Beavo (2006)

--- unknown, *n.s.* no substrate (no hydrolysis detected)

[a]Reinecke et al. (2011)

[b]Monzel et al. (2014)

projects should aim at completing our knowledge by performing unbiased enzyme kinetic studies using all cNMPs as substrates, regardless of the traditional PDE classification in cAMP-, cGMP- and dual-"specific" PDEs.

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3′,5′-cIMP as Potential Second Messenger in the Vascular Wall

Susan W.S. Leung, Yuansheng Gao, and Paul M. Vanhoutte

Contents

Abstract

Traditionally, only the 3′,5′-cyclic monophosphates of adenosine and guanosine (produced by adenylyl cyclase and guanylyl cyclase, respectively) are regarded as true "second messengers" in the vascular wall, despite the presence of other cyclic nucleotides in different tissues. Among these noncanonical cyclic nucleotides, inosine 3′,5′-cyclic monophosphate (cIMP) is synthesized by soluble guanylyl cyclase in porcine coronary arteries in response to hypoxia, when the enzyme is activated by endothelium-derived nitric oxide. Its production is associated with augmentation of vascular contraction mediated by stimulation of Rho kinase. Based on these findings, cIMP appears to meet most, if not all, of the criteria required for it to be accepted as a "second messenger," at least in the vascular wall.

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Keywords
Hypoxia • Inosine 3′,5′-cyclic monophosphate • Magnesium ions • Rho kinase • Soluble guanylyl cyclase

Abbreviations

AMP	Adenosine 5′-monophosphate
ATP	Adenosine 5′-triphosphate
cAMP	Adenosine 3′,5′-cyclic monophosphate
cGMP	Guanosine 3′,5′-cyclic monophosphate
cIMP	Inosine 3′,5′-cyclic monophosphate
GMP	Guanosine 5′-monophosphate
GTP	Guanosine 5′-triphosphate
IMP	Inosine 5′-monophosphate
ITP	Inosine 5′-triphosphate
Mg^{2+}	Magnesium ions
Mn^{2+}	Manganese ions
MYPT1	Myosin phosphatase target subunit 1
ODQ	1*H*-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one
sGC	Soluble guanylyl cyclase

1 Introduction

Over the years, 3′,5′-cyclic monophosphates of different nucleosides, including those of adenosine (cAMP), cytidine, guanosine (cGMP), inosine (cIMP), thymidine, and uridine, have been detected in different cells and tissues (Ferguson and Price, 1973a; Bloch, 1975; Garbers et al. 1975; Cech and Ignarro, 1978; Newton et al. 1986; Göttle et al. 2010; Hartwig et al. 2014; Bähre et al. 2015). Among these 3′,5′-cyclic monophosphates, cAMP and cGMP are well recognized as "second messengers," based on the identification of the physiological "first messengers" stimulating their synthesis [prostaglandins and nitric oxide, respectively], of the enzymes responsible for their production [adenylyl cyclase and guanylyl cyclase, respectively] and degradation [their respective selective phosphodiesterases], and of their cellular targets [their respective selective protein kinases] and functions [e.g., vasodilatation and inhibition of platelet reaction] (Hardman et al. 1971a, b; Moncada and Vane, 1979; Mustard et al. 1980; Ignarro et al. 1987; Goy, 1991; Ritchie et al. 2009). By contrast, cIMP is considered to be a "noncanonical" cyclic nucleotide. Despite the early discovery of its production in the toad bladder, sea urchin sperm, and rat lungs (Ferguson and Price, 1973a; Garbers et al. 1975), a role

for cIMP as a second messenger has not been established. Two independent studies demonstrate that cIMP can be a major product of the activity of soluble guanylyl cyclase (sGC) under certain conditions (Beste et al. 2012; Chen et al. 2014) and that it mediates the hypoxia-induced contractions of isolated porcine coronary arteries (Chen et al. 2014), suggesting a physiological role for this "noncanonical" cyclic nucleotide. The present chapter provides an overview of the synthesis and metabolism of cIMP and discusses the evidence supporting its role as a second messenger.

2 Synthetic Pathways Leading to the Formation of cIMP

Several pathways can lead to the production of cIMP. As early as in 1973, cIMP was detected in toad bladder homogenates as the product of the deamination of cAMP (Ferguson and Price, 1973a, b). The enzyme catalyzing the deamination, adenosine deaminase, is not selective for cAMP; it also deaminates other adenine derivatives, including adenosine, adenosine 5′-phosphate, and deoxyadenosine derivatives (Wolfenden et al. 1967; Fig. 1). A deaminase selective for cAMP leading to the formation of cIMP was identified in the human pathogen, *Leptospira interrogans* (Goble et al. 2013); whether or not such a selective cAMP deaminase exists in mammals remains to be determined.

It is also possible that cIMP could be formed from cGMP, in view of the structural similarity of the two cyclic nucleotides. While a direct conversion of cGMP to cIMP has not been reported, the metabolite of cGMP, guanosine 5′-monophosphate (GMP), can undergo reductive deamination to produce inosine 5′-monophosphate (IMP; Magasanik and Karibian, 1960; Mackenzie and Sorensen, 1973; Barankiewicz and Cohen, 1984; Deng et al. 2002). This reaction requires nicotinamide adenine dinucleotide phosphate and is catalyzed by GMP reductase, which is present in many organisms including bacteria, parasites, rats, and humans (Mager and Magasanik, 1960; Mackenzie and Sorensen, 1973; Spector and Jones, 1982; Salvatore et al, 1998). Only a small amount of GMP is deaminated reductively to IMP; GMP is mainly converted through GMP kinase to guanosine 5′-triphosphate (GTP) or through 5-′-nucleotidase to guanosine (Weber et al. 1992; Traut, 1994). IMP is also formed as the result of the deamination of adenosine 5′-monophosphate (AMP), de novo synthesis from phosphoribosyl pyrophosphate 5-phospho-α-D-ribose 1-diphosphate, conversion

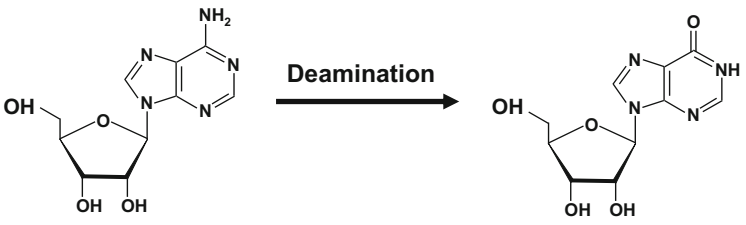

Fig. 1 Deamination of adenosine to inosine

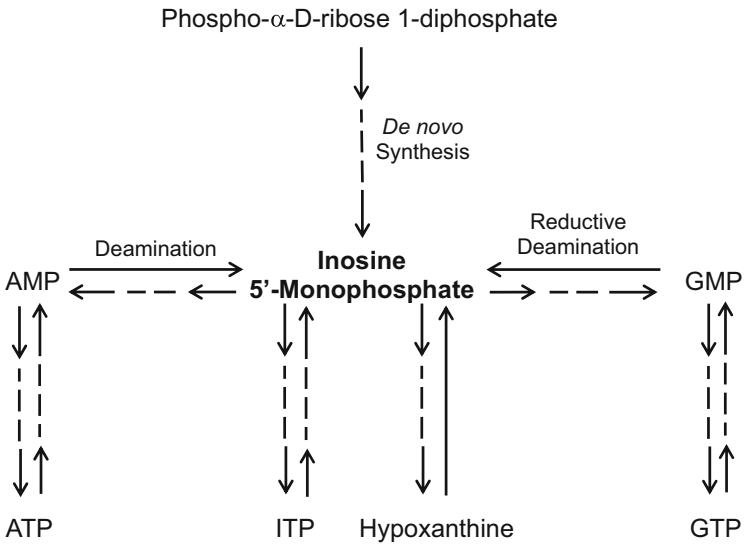

Fig. 2 Synthetic and metabolic pathways for inosine 5′-monophosphate (based on Weber et al. 1992 and Zhao et al. 2015). *AMP* adenosine 5′-monophosphate, *ATP* adenosine 5′-triphosphate, *GMP* guanosine 5′-monophosphate, *GTP* guanosine 5′-triphosphate, *ITP* inosine 5′-triphosphate

from inosine 5′-triphosphate (ITP) by ITP pyrophosphatase, and from hypoxanthine by hypoxanthine-guanine phosphoribosyltransferase (Vanderheiden, 1975; Weber et al. 1992; Torrecilla et al. 2001; Barsotti et al. 2003; Zhao et al. 2015; Fig. 2). It serves as a purine nucleotide precursor to be converted to AMP and GMP; this process is an important mechanism for the maintenance of the intracellular level of purine nucleotides (Mager and Magasanik, 1960; Weber et al. 1992; Zoref-Shani et al. 1995; Torrecilla et al. 2001). Nevertheless, IMP may be phosphorylated by kinases to form ITP (Vanderheiden, 1979; Sakumi et al. 2010).

Using radioactively labeled ITP as the substrate, the occurrence of de novo synthesis of cIMP (Fig. 3) has been confirmed in various rat tissues, including the liver, heart, kidney, brain, intestine, spleen, testis, and lung (Garbers et al. 1975; Newton et al. 1986). The presence of the synthetic pathway seems to indicate a physiological role for the cyclic nucleotide, rather than it being merely a metabolite of cAMP. However, a selective enzyme responsible for the conversion of ITP to cIMP has not been identified. Adenylyl cyclase may be responsible for the conversion, as suggested by the observation that in the toad bladder the production of both cAMP and cIMP can be stimulated by the activator of adenylyl cyclase, oxytocin (Ferguson and Price, 1973a). However, this proposal is argued against by the findings that the formation of cIMP (and cGMP) in the particulate fraction of sea urchin sperm persists after inhibition of adenylyl cyclase activity by heat (30°C for 40 min) and that homogenates of rat lungs, which lack adenylyl cyclase activity,

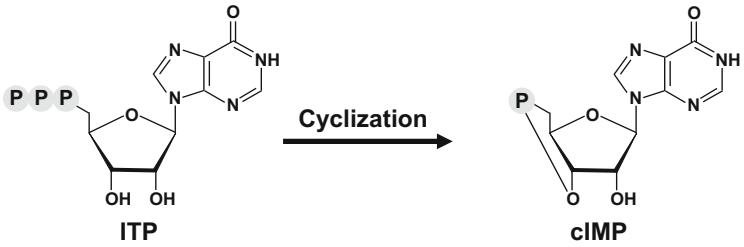

Fig. 3 Conversion of inosine 5′-triphosphate (ITP) to inosine 3′,5′-cyclic monophosphate (cIMP)

can convert ITP to cIMP (Garbers et al. 1975). The purified adenylyl cyclase toxin edema factor from *Bacillus anthracis* can use ITP as substrate to produce cIMP but with very low efficacy (Göttle et al. 2010). On the other hand, correlation studies examining the degree of cGMP and cIMP formation from their respective precursor nucleotide triphosphates in particulate fractions of sea urchin sperm [after exposure to different temperatures (30 to 40°C for 20 to 80 min) or incubation with various cations (barium, calcium, lanthanum, magnesium, manganese, strontium) in the presence of trypsin for five minutes at 30°C to eliminate adenylyl cyclase activity] prompted the conclusion that both reactions may be catalyzed by the same enzyme (Garbers et al. 1975). Direct evidence supporting the ability of sGC to produce cIMP came from experiments using purified sGC in a baculovirus/Sf9 system (Hoenicka et al. 1999) with the nitric oxide donor sodium nitroprusside as the stimulator (Beste et al. 2012). Moreover, in freshly isolated porcine coronary arterial smooth muscle, the level of cIMP can be increased by the nitric oxide donor diethylenetriamine NONOate; this increase is abolished by the sGC inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ; Chen et al. 2014). Taken in conjunction the available evidence suggests that sGC is the natural enzyme responsible for the generation of cIMP.

3 Stimulation of cIMP Production

While sGC catalyzes the conversion of ITP to cIMP, this reaction is about three- to tenfold less effective than that leading to cGMP production in the presence of manganese ions (Mn^{2+}; Garbers et al. 1975; Beste et al. 2012), which appear to be the divalent cation required for sGC activity in vivo (Beste and Seifert, 2013; Bähre et al. 2014). The absence of a selective "inosinyl cyclase" prompts the question whether or not endogenous production of cIMP occurs. To ascertain that the experimental finding of the cellular production of cIMP is not due to the nonselective nature of sGC, physiological or pathological stimuli initiating the synthesis of cIMP must be identified. In the examination of the hypoxia (PO_2, 25–30 mmHg)-induced contraction of isolated porcine coronary arteries, a signaling pathway has been defined, whereby the activation of sGC by endothelium-derived nitric oxide is required to facilitate the contractile process, but not the production of cGMP, the

Table 1 Michaelis–Menten kinetic parameters of sGC for GTP and ITP (Beste et al. 2012)

	GTP	ITP
Mg^{2+} as cofactor		
K_m (μM)	119.1 ± 13.7	796 ± 90
V_{max} (μmol min^{-1} mg^{-1})	2.2 ± 0.5	4.9 ± 0.1
Mn^{2+} as cofactor		
K_{m1} (μM)	14.2 ± 1.6	6.6 ± 1.9
V_{max1} (μmol min^{-1} mg^{-1})	4.1 ± 0.15	1.4 ± 0.1
K_{m2} (μM)	4819 ± 552	13,189
V_{max2} (μmol min^{-1} mg^{-1})	13.5 ± 0.7	3.4 ± 1.5

Kinetics of formation of cIMP or cGMP by soluble guanylyl cyclase (sGC) in the presence of sodium nitroprusside (10^{-4} M) and Mn^{2+} [NTP/Mn^{2+} and Mn^{2+} (3×10^{-3} M)] or Mg^{2+} [NTP/Mg^{2+} and Mg^{2+} (3×10^{-3} M)]. The enzyme (0.1–20 ng of sGC per tube) was incubated with sodium nitroprusside and various concentrations (2×10^{-6} to 7.5×10^{-3} M) of the respective NTP

cGMP guanosine 3′,5′-cyclic monophosphate, *cIMP* inosine 3′,5′-cyclic monophosphate, *GTP* guanosine 5′-triphosphate, *ITP* inosine 5′-triphosphate, K_m concentration required to produce half-maximal rate of activity, Mg^{2+} magnesium ions, Mn^{2+} manganese ions, *NTP* nucleotide 5′-triphosphate, *SEM* standard error of mean, *sGC* soluble guanylyl cyclase, V_{max} maximal rate of activity

"canonical" product of the enzyme (De Mey and Vanhoutte, 1983; Rubanyi and Vanhoutte, 1985; Gräser and Vanhoutte, 1991; Pearson et al. 1996; Chan et al. 2011). High-performance liquid chromatography tandem mass spectrometry revealed an increased level of cIMP, but not of cGMP or other cyclic nucleotides, in homogenates of coronary arteries exposed to hypoxia; this increase was sensitive to the prototypical inhibitor of sGC, ODQ (Chen et al. 2014). Therefore, hypoxia appears to be a natural stimulus for sGC to produce cIMP instead of cGMP.

Over the years, cGMP has been considered to be the only physiologically relevant product synthesized by sGC (Waldman and Murad, 1987; Friebe and Koesling 2009), despite indications of cIMP production by the enzyme in various rat tissues (Garbers et al. 1975; Newton et al. 1986). In experiments with purified sGC stimulated by sodium nitroprusside, GTP (the precursor of cGMP) and ITP bound to both an allosteric site of the enzyme, where they stabilize it in its active state, and the catalytic site, where they are converted to their cyclic monophosphate forms (Beste et al. 2012). The binding affinity (reflected by the Michaelis–Menten kinetic K_m value) of GTP for the catalytic site is higher than that of ITP, although the maximal rate of cIMP formation is greater than that of cGMP production, with magnesium ions (Mg^{2+}, 3×10^{-3} M) as the cofactor (Beste et al. 2012; Table 1). However, the activity of sGC is greater in the presence of Mn^{2+} (3×10^{-3} M; Hardman and Sutherland, 1969), under which condition the binding affinity of ITP is higher than that of GTP, but the maximal rate of cIMP formation is less than that of cGMP (Beste et al. 2012). Nevertheless, the activity of the enzyme under basal conditions is consistently higher with GTP than with ITP as the substrate [about 18-fold in the presence of Mn^{2+} and fivefold in the presence of Mg^{2+} (Beste

Table 2 Conditions favoring the generation of inosine 3′,5′-cyclic monophosphate by soluble guanylyl cyclase

Factors	Effects	References
Nitric oxide	Increase the relative enzymatic activity for using GTP to ITP as the substrate (in the presence of Mn^{2+}, guanylyl cyclase activity increased by fivefold; inosinyl cyclase activity by 33-fold; in the presence of Mg^{2+}, guanylyl cyclase activity increased by 100-fold; inosinyl cyclase activity by 254-fold)	Beste et al. (2012)
Mg^{2+}	Increase the rate of production of cIMP	Beste et al. (2012)
ITP	Provide the substrate for enzymatic activity	Garbers et al. (1975)
ITP	Inhibit guanylyl cyclase activity	Chang et al. (2005)

GTP guanosine 5′-triphosphate, *ITP* inosine 5′-triphosphate, Mg^{2+} magnesium ions, Mn^{2+} manganese ions

et al. 2012)]. When sGC is activated by sodium nitroprusside, the relative activity of the enzyme using GTP rather than ITP as substrate is reduced to about twofold with either Mn^{2+} or Mg^{2+} as cofactor (Beste et al. 2012), suggesting that nitric oxide stimulates sGC to have greater "inosinyl cyclase" than "guanylyl cyclase" activity. Moreover, the "guanylyl cyclase" activity of purified sGC is inhibited by ITP more effectively when the enzyme is stimulated with nitric oxide (Chang et al. 2005). Table 2 summarizes the conditions that favor the production of cIMP by sGC.

Under normal conditions, little or no cIMP is generated by the inosinyl cyclase activity of sGC; this is likely due to a low intracellular level of substrate (Hardman et al. 1971b; Behmanesh et al. 2009; Beste et al. 2013; Chen et al. 2014). ITP is produced mainly by the deamination of ATP and to a lesser degree by the phosphorylation of inosine mono- and diphosphates (Behmanesh et al. 2009; Sakumi et al. 2010). The intracellular level of ITP under normal conditions is low to avoid competition with ATP and GTP for adenylyl cyclase and sGC, respectively, which would result in inhibition of physiological reactions that are important for normal cellular activity and growth (Auclair et al. 1990; Muraoka et al. 1999; Vormittag and Brannath, 2001; Behmanesh et al. 2009). The low intracellular level of ITP is achieved by the activity of the enzyme ITP pyrophosphatase, which hydrolyzes ITP to its mono- and diphosphate forms (Fig. 4). Therefore, in addition to providing conditions facilitating the production of cIMP (e.g., by increasing the intracellular level of Mg^{2+}), an increased intracellular level of ITP is also required in order to change the reaction product of sGC from cGMP to the noncanonical cyclic nucleotide.

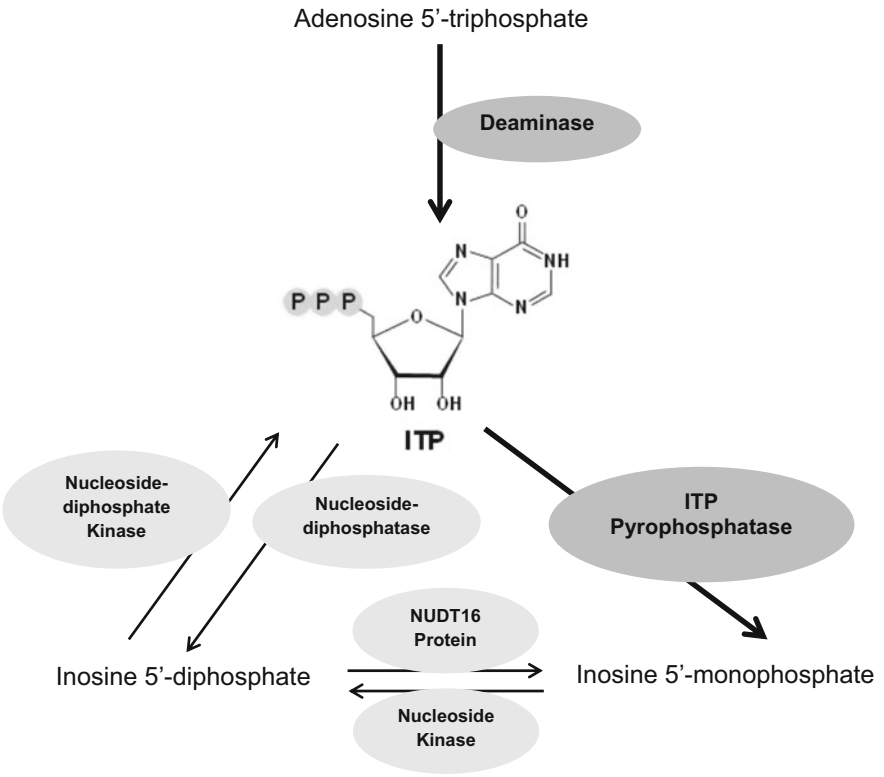

Fig. 4 Synthetic and metabolic pathways for inosine 5′-triphosphate (ITP, based on Sakumi et al. 2010)

4 Metabolic Pathways of cIMP

To serve a role in signal transduction, cIMP would need to return rapidly to its basal level after producing its biological effect(s). Like the canonical cyclic nucleotides, the degradation of cIMP appears to be achieved by phosphodiesterase activity, as observed in homogenates of various tissues, in particular the liver of the rat (Miller et al. 1976; Newton et al. 1986). It is unclear whether or not a selective cIMP phosphodiesterase exists. The inhibitory effect by cIMP on the degradation of other cyclic nucleotides by phosphodiesterase(s) [reported in homogenates of human lung, rabbit lung, bovine heart, cat heart, and rat brain (Harris et al. 1973; Miller et al. 1976; Bergstrand et al. 1978)] suggests that it indeed binds to the catalytic site of phosphodiesterase(s). Moreover, cIMP can be hydrolyzed by a supposedly cGMP-selective phosphodiesterase from *Dictyostelium discoideum* (van Haastert and van Lookeren Campagne 1984) and by partially purified human recombinant phosphodiesterases, expressed in Sf9 insect cells, despite their assumed preference

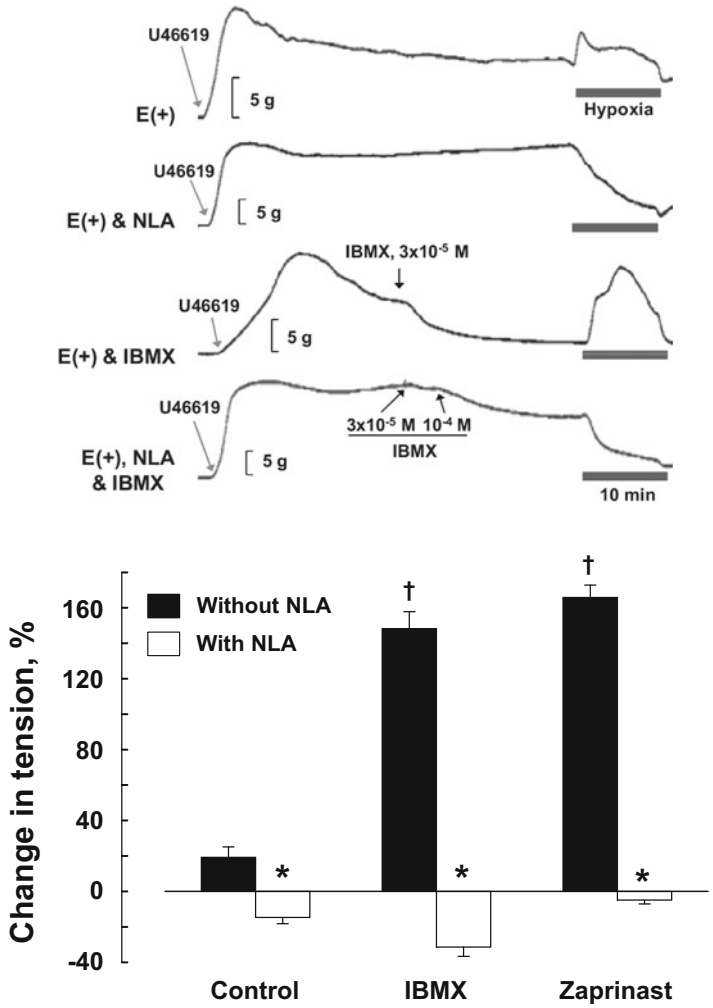

Fig. 5 Effects of phosphodiesterase inhibitors on hypoxia-induced contraction (Courtesy of Zhengju Chen and Yansheng Gao, unpublished observations). *Upper*: Original tracing showing the contraction of porcine coronary arteries with endothelium [E(+)]. Arteries were contracted with U46619 (a stable thromboxane A_2 analogue, 2×10^{-8} M to 3×10^{-7} M) to a similar tension level, in the absence (control) or presence of IBMX (nonselective phosphodiesterase inhibitor, 3×10^{-5} M) and zaprinast [selective inhibitor of phosphodiesterase 5 (cGMP-selective), 10^{-5} M] with and without nitro-L-arginine (nitric oxide synthase inhibitor; NLA, 10^{-4} M). During the sustained contraction, arteries were exposed to hypoxia. *Lower*: The effect of hypoxia was calculated by the area under the isometric tension trace of the first three minutes of hypoxia over that of the last three minutes of the contraction to U46619 prior to hypoxia. *Asterisk*, significantly different from respective group without NLA; *dagger*, significantly different from respective control group ($P < 0.05$). Data are shown as means ± SEM; $n = 6$ for each group

for hydrolyzing cAMP (phosphodiesterases 3A and 3B) and cGMP (phosphodiesterases 1B and 5A) (Reinecke et al. 2011). In porcine coronary arteries, phosphodiesterase inhibitors potentiate the response to hypoxia, attributed to the formation of cIMP (Fig. 5)

5 Cellular Targets of cIMP

Protein binding sites for cIMP, with a range of 10^{-10} to 10^{-8} mol mg^{-1} protein, have been detected in different tissues of the rat (Newton et al. 1986). In view of structural similarity, cIMP is proposed to bind to cAMP- and cGMP-binding proteins. These include not only the metabolizing proteins, phosphodiesterases (Harris et al. 1973; Bergstrand et al. 1978), but also the catabolite gene activator in *Escherichia coli* (Anderson et al. 1972), the transporter that preferentially takes up cAMP in Malpighian tubule cells of *Drosophila melanogaster* (Riegel et al. 1998) and cAMP-dependent protein kinase in the mammalian central nervous system (Gundlach and Urosevic, 1989). In addition, cIMP can mimic the biological effects of cAMP, albeit with reduced efficiency, in *Dictyostelium discoideum* (Konijn et al. 1969). Direct measurements of protein kinase activity using radioactively labeled ATP indicate that cIMP activates cAMP-dependent protein kinase purified from human polymorphonuclear leucocytes (Tsung et al. 1972) and bovine heart and brain (Kuo et al. 1974; Miller et al. 1976), as well as cGMP-dependent protein kinase purified from lobster tail muscle (Kuo et al. 1974; Miller et al. 1976). Although the efficacy of cIMP in activating these proteins is similar to those of the canonical cyclic nucleotides, its potency is about ten- to 100-fold less (Kuo et al. 1974; Miller et al. 1976; Wolter et al. 2011). Since significant activation of these protein kinases only occurs at micromolar concentrations of cIMP (Wolter et al. 2011; Table 3), it appears unlikely that they are the cellular targets for cIMP to exert biological actions. However, since cIMP binds to these sites at much lower concentrations than those required for their activation (Kuo et al. 1974; Newton et al. 1986), it may inhibit (by competing with the same binding sites on protein kinases) the biological effects of cAMP and/or cGMP (Fig. 6).

By analogy, cIMP can compete with the canonical cyclic nucleotides for the catalytic sites of the phosphodiesterases, thus leading to the enhancement of the biological effects of the latter (Fig. 6; Harris et al. 1973; Miller et al. 1976; Bergstrand et al. 1978). Besides catalytic sites, phosphodiesterases contain other binding sites for cyclic nucleotides, termed allosteric sites, which appear to be involved in the regulation of the catalytic activity of the enzyme (Turko et al. 1998; Wyatt et al. 1998; Corbin et al. 2000; Meima et al. 2003; Heikaus et al. 2009). The activities of cAMP- and cGMP-selective phosphodiesterases isolated from *Dictyostelium discoideum* were increased when their allosteric binding sites were occupied by the respective cyclic nucleotides (van Haastert et al. 1982; Meima et al. 2003). In these studies, the allosteric sites of the selective phosphodiesterase had the "specificity" for the respective cyclic nucleotides, thus suggesting that these sites can serve as a negative feedback mechanism to regulate the intracellular level

Table 3 Range of concentrations of inosine 3′,5′-cyclic triphosphate for activations of various protein kinases

Protein kinases	Concentrations of cyclic nucleotide (M)	EC_{50} value (M)	References
cAMP-dependent protein kinase			
Isolated from bovine heart	10^{-8} to 10^{-5} [cAMP, 10^{-8} to 10^{-6}]	2×10^{-7} [3×10^{-8}]	Kuo et al. (1974)
PKA Cα2/RI α2 isozymes	10^{-7} to 10^{-6} [cAMP, 10^{-8} to 10^{-7}]	10^{-6} [7×10^{-8}]	Wolter et al. (2011)
PKA Cα2/RII α2 isozymes	10^{-7} to 10^{-4} [cAMP, 10^{-8} to 10^{-6}]	4×10^{-6} [10^{-7} M]	Wolter et al. (2011)
cGMP-dependent protein kinase			
Isolated from lobster tail muscle	10^{-7} to 10^{-4} [cGMP, 10^{-8} to 10^{-5}]	8×10^{-6} [5×10^{-8}]	Kuo et al. (1974)
PKGIα isozymes	10^{-6} to 10^{-4} [cGMP, 10^{-8} to 10^{-6}]	2×10^{-5} [10^{-7}]	Wolter et al. (2011)
Rho kinase (porcine coronary arteries)	10^{-7} to 10^{-6}	ND	Chen et al. (2014)

cAMP adenosine 3′,5′-cyclic monophosphate, *cGMP* guanosine 3′,5′-cyclic monophosphate, *EC_{50} value* concentration that cause half-maximal stimulation of the protein kinase, *ND* not determined

of cAMP and cGMP. Binding of cAMP to the allosteric sites of phosphodiesterases leading to increased hydrolysis of cGMP, and vice versa, also occurs (Beavo et al. 1970; Moss et al. 1977; Zaccolo and Movsesian, 2007; Heikaus et al. 2009). In addition, cIMP either can (Moss et al. 1977; Liu et al. 2002; Okada and Asakawa, 2002) or cannot (van Haastert et al. 1982) bind to the allosteric sites of cGMP-selective phosphodiesterase, and such binding may (Moss et al. 1977) or may not (Okada and Asakawa, 2002) lead to the activation of the enzyme. These discrepancies may be due to different experimental conditions and/or different enzymatic isoforms/families involved. At least eleven phosphodiesterase families have been identified; they differ in the tissue distribution, substrate selectivity, and also regulatory mechanisms (Zaccolo and Movsesian, 2007; Heikaus et al. 2009; Francis et al. 2011). Phosphodiesterases are also regulated by their phosphorylation state, and some of them can be phosphorylated by cAMP- and/or cGMP-selective protein kinases (Sette and Conti 1996; Turko et al. 1998; Wyatt et al. 1998; Corbin et al. 2000; Francis et al. 2011; Byrne et al. 2015). Therefore, cIMP may modulate the action of phosphodiesterases by competing with the canonical cyclic nucleotides for their binding sites on protein kinases. As the phosphorylation of phosphodiesterases may depend on the binding of the canonical cyclic nucleotides to allosteric sites (Turko et al. 1998; Corbin et al. 2000; Francis et al. 2011), the potential effect of cIMP on the activity of phosphodiesterases is rather difficult to predict. Clearly, a comprehensive study on the regulatory role of cIMP on different phosphodiesterase families is warranted in order to determine how this cyclic nucleotide affects cellular functions determined by the intracellular levels of cAMP and/or cGMP.

In addition to a possible interference with cAMP and cGMP signaling, cIMP may activate its own downstream signaling pathway. The production of cIMP in

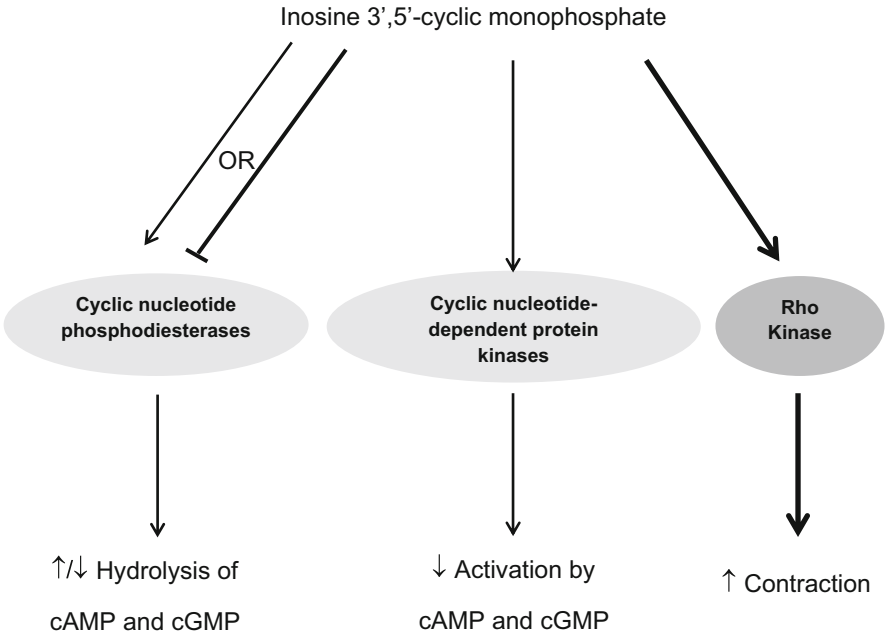

Fig. 6 Possible biological effects of inosine 3′,5′-cyclic monophosphate (cIMP). cIMP binds to the catalytic sites and inhibits cyclic nucleotide phosphodiesterases, thereby reducing the hydrolysis of adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) and thus enhancing the activities of these cyclic nucleotides. On the other hand, it may bind to the allosteric sites to activate the cyclic nucleotide phosphodiesterases (either directly or promote the phosphorylation of the enzyme) to increase the hydrolysis of the canonical cyclic nucleotides. Depending on the isoforms of the phosphodiesterase, increased phosphorylation may result in inhibition of the enzyme. cIMP may also compete with cAMP and cGMP for their respective cyclic nucleotide-dependent protein kinases thus inhibiting their effects; this may also lead to reduced phosphorylation of phosphodiesterases. Activation of Rho kinase by cIMP to enhance vascular smooth muscle contraction has been reported; however, it is not clear whether or not cIMP directly binds to this enzyme

vascular tissues during hypoxia is associated with contraction, which is more pronounced when the vascular tone of the blood vessels is increased first by other contracting agents (i.e., augmentation of contraction; Gräser and Vanhoutte, 1991; Chan et al. 2011; Chen et al. 2014). Such augmentation of contraction is unlikely to be the consequence of interference with the effects of the canonical cyclic nucleotides since it is (1) not affected by inhibitors of cAMP- and cGMP-dependent protein kinases (Chan et al. 2011), (2) not associated with an increased intracellular level of either cAMP or cGMP (Chan et al. 2011; Chen et al. 2014), and (3) potentiated by inhibitors of phosphodiesterases (Gräser and Vanhoutte, 1991; Fig. 5). The cIMP-mediated hypoxic augmentation is reduced by Rho kinase inhibitors (Y27632 and HA1077; Chan et al. 2011; Chen et al. 2014), suggesting the involvement of this enzyme, which enhances contraction of vascular smooth muscle cells by decreasing the phosphorylation of myosin phosphatase target subunit 1 (MYPT1) at Thr^{696} and Thr^{853}, resulting in reduced myosin light-chain

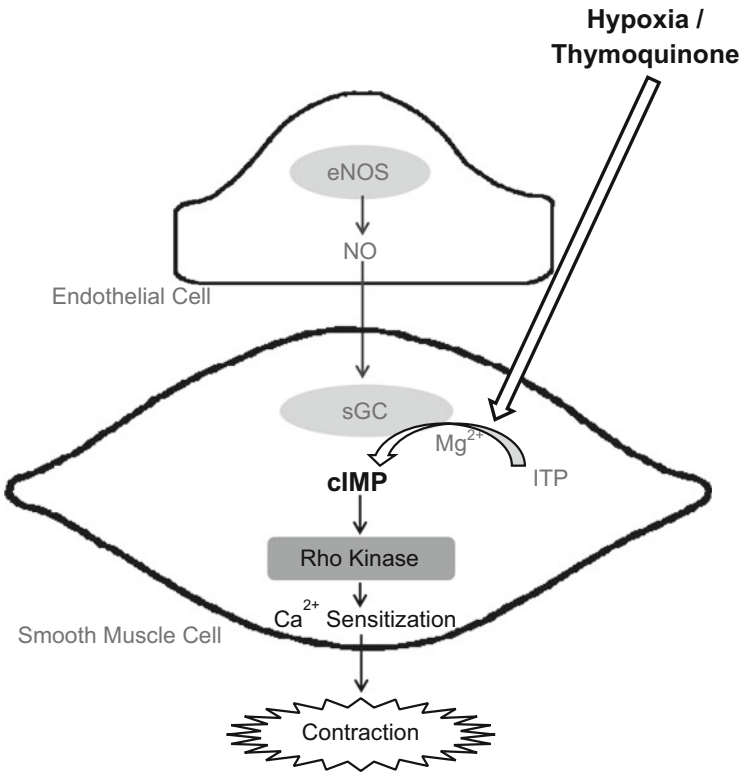

Fig. 7 Proposed signaling pathway for vascular contraction that involves inosine 3′,5′-cyclic monophosphate (cIMP) as the second messenger. In the presence of nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) and activating soluble guanylyl cyclase (sGC), hypoxia (or thymoquinone) increases the intracellular level of ITP and perhaps also that of magnesium ions (Mg^{2+}) to facilitate the biased production of cIMP. The cyclic nucleotide, either directly or indirectly, activates Rho kinase, which, in turn, inhibits myosin light-chain phosphatase to cause the sensitization of the contractile myofilaments to calcium (Ca^{2+}), thereby enhancing contraction of the vascular smooth muscle cells

phosphatase activity and hence facilitated interaction of the contractile proteins (Kimura et al. 1996; Fu et al. 1998; Nagumo et al. 2000; Kitazawa et al. 2003; Tsai and Jiang, 2006). Indeed, in isolated porcine coronary arteries, cIMP [in submicromolar concentrations, which are likely to be physiologically relevant to judge from the concentrations of canonical cyclic nucleotides needed for activation of their respective protein kinases (Beavo et al. 1970; Hardman et al. 1971b; Harris et al. 1973)], increases the activity of Rho kinase and the phosphorylation of MYPT1 at Thr^{853} (Chen et al. 2014; Table 3). Therefore, the signaling pathway involving cIMP and leading to augmented contractions of vascular smooth muscle cells appears to rely on Rho kinase and myosin phosphatase (Fig. 7); however, it remains to be determined whether or not cIMP directly binds to Rho kinase to achieve such activation.

6 Physiological and/or Pathological Role of cIMP

The presence of the synthetic and metabolic pathways, coupled with the identification of a downstream signaling target, suggests that cIMP can serve as a mediator for cellular responses. At present, the only identified physiologically relevant stimulus for cIMP production is hypoxia (Chen et al. 2014; Gao et al. 2015; Gao and Vanhoutte, 2014), which in large systemic arteries is considered to be a pathological condition. The generation of cIMP requires the activation of sGC by nitric oxide (Chen et al. 2014). Therefore, in the detrimental vascular hypoxic response, cIMP appears to be the "second messenger" of the "first messenger" nitric oxide (Chen et al. 2014). A switch of a physiologically vasodilator signal [endothelium-derived nitric oxide] to cause vasoconstriction under pathological conditions is not new. Thus, it is well established that under normal conditions, endothelium-derived prostacyclin activates adenylyl cyclase to produce the second messenger cAMP for vasodilatation (Moncada and Vane, 1979; Ignarro et al. 1987); however, in blood vessels of hypertensive or diabetic animals, this "first messenger" activates the thromboxane-prostanoid receptors causing vascular contraction (Gluais et al. 2005; Tang and Vanhoutte, 2009; Félétou et al. 2011; Zhu et al. 2014). By analogy, the production of a vasoconstrictor second messenger instead of the vasodilator cGMP by endothelium-derived nitric oxide may be initiated by the different intracellular environment caused by hypoxia. As mentioned above, the presence of Mg^{2+} as cofactor promotes the production of cIMP from ITP by sGC (Beste et al. 2012). While the intracellular concentration of free Mg^{2+} remains relatively constant under normal conditions, hypoxia causes rapid changes in intracellular Mg^{2+} concentration in sensory neurons (Henrich and Buckler, 2008). The increased intracellular level of Mg^{2+} may be the consequence of a depletion in cellular ATP, which normally is present in the form of Mg^{2+}-ATP due to the its high affinity for the divalent ion (Romani and Scarpa, 2000; Henrich and Buckler, 2008). If such changes were to occur in vascular smooth muscle cells, cIMP would be the major mediator produced subsequent to biased sGC activation, provided the intracellular level of substrate ITP (which can be produced by the deamination of ATP) is augmented. As a matter of fact, in isolated porcine coronary arteries, the amount of ITP, initially undetectable, increases upon exposure to hypoxia (Chen et al. 2014). Whether or not this increase is the consequence of increased production, reduced degradation, or both, of ITP requires further investigation.

Under hypoxia, the switch of the production of vasodilator second messenger cGMP to the vasoconstrictor cIMP by sGC, if it were to occur in the pulmonary circulation, may serve as an adaptive feedback measure to divert blood to regions of the lung with adequate oxygen supply, thereby preserving the ventilation to perfusion ratio. However, if such biased activity of sGC occurs in the coronary circulation, a transient reduction in blood oxygen level, comparable to those observed under conditions of sleep apnea, would precipitate detrimental coronary spasm leading to myocardial ischemia (Pearson et al. 1996). This phenomenon may explain the increased risk of coronary diseases in patients with sleep apnea

(Budhiraja et al. 2010; Kasai et al. 2012). There are other pathological conditions that are associated with local or systemic transient hypoxia, as is the case, for example, in coronary arteries with atherosclerotic plaques (resulting in reduced arterial diameter and hence decreased blood flow and oxygen supply to the myocardium) or rapid ascent to high altitude (reduced atmospheric partial pressure with reduced availability of oxygen for diffusion into the blood). If cIMP were released in response to local hypoxia under these conditions, it may serve as a second messenger responsible for detrimental outcomes.

In addition to hypoxia, the natural product, thymoquinone (which is a biologically active constituent of *Nigella sativa*; Ragheb et al. 2009), stimulates cIMP production in a nitric oxide-dependent manner in isolated porcine coronary arteries and rat aortae and mesenteric arteries, leading to augmentation of contraction with pharmacological characteristics comparable to those of hypoxia-induced facilitation (namely, inhibition by endothelium removal, dependency on activation of sGC without augmented cGMP production, and involvement of Rho kinase; Leung et al. 2013; Detremmerie et al. 2014). These pharmacological observations further confirm the potential role of biased activity of sGC initiating a cIMP signaling pathway, whereby this noncanonical cyclic nucleotide serves as a second messenger to exacerbate vascular contraction (Fig. 7).

7 Conclusion

In summary, evidence is emerging which suggests that cIMP can be considered as a second messenger (Gao and Vanhoutte, 2014; Seifert et al. 2015): (1) The intracellular level of cIMP increases in response to hypoxia [a physiologically relevant stimulus], as a result of the biased activation of sGC [a second messenger-generating enzyme] following the production of endothelium-derived nitric oxide [the first messenger] (Chen et al. 2014). (2) The increase is associated with the activation of Rho kinase and the subsequent enhanced phosphorylation of MYPT1 [specific effector system] leading to contraction of vascular smooth muscle [a defined cell function] (Chen et al. 2014). (3) The biological effects [hypoxic augmentation mediated by the activation of Rho kinase] can be reproduced experimentally not only by exogenous cIMP [albeit its high concentrations since only approximately 1% of the cyclic nucleotide can diffuse into the cells (Chen et al. 2014)] but also by its precursor ITP (Chen et al. 2014). (4) cIMP can be degraded by phosphodiesterases [inactivation mechanisms] (Miller et al. 1976; van Haastert and van Lookeren Campagne 1984; Newton et al. 1986; Reinecke et al. 2011), and inhibition of these enzymes results in potentiation of hypoxia-induced augmentation of contraction (Gräser and Vanhoutte, 1991; Fig. 5).

A critical review of the evidence indicates that, although Rho kinase appears to be the downstream effector of cIMP in porcine coronary vascular smooth muscle, the cellular protein to which this cyclic nucleotide binds directly to achieve its biological effect remains to be identified. Moreover, the enzymes responsible for the synthesis and hydrolysis of cIMP [sGC and phosphodiesterases] are not specific

for this noncanonical cyclic nucleotide. The synthesis of cIMP by sGC apparently requires the presence of both nitric oxide and increased levels of ITP and perhaps also an increased intracellular Mg^{2+}; the combination of those cellular changes explains the biased production of cIMP and hence the subsequent cellular responses that it causes. To actually label cIMP as a second messenger, further experiments are warranted [e.g., to confirm the natural occurrence of cIMP in mammalian cells using extremely precise high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (Hartwig et al. 2014; Bähre et al. 2015) and to determine whether or not cIMP binds directly to Rho kinase or its upstream signal Rho protein]; the current evidence obtained so far from both biochemical assays and functional studies, nonetheless, appears convincing.

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Discovery and Roles of 2′,3′-cAMP in Biological Systems

Edwin K. Jackson

Contents

Abstract

In 2009, investigators using ultra-performance liquid chromatography-tandem mass spectrometry to measure, by selected reaction monitoring, 3′,5′-cAMP in the renal venous perfusate from isolated, perfused kidneys detected a large signal at the same m/z transition (330 → 136) as 3′,5′-cAMP but at a different retention time. Follow-up experiments demonstrated that this signal was due to a positional isomer of 3′,5′-cAMP, namely, 2′,3′-cAMP. Soon thereafter, investigative teams reported the detection of 2′,3′-cAMP and other 2′,3′-cNMPs (2′,3′-cGMP, 2′,3′-cCMP, and 2′,3′-cUMP) in biological systems ranging from bacteria to plants to animals to humans. Injury appears to be the major stimulus for the release of these unique noncanonical cNMPs, which likely are formed by

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the breakdown of RNA. In mammalian cells in culture, in intact rat and mouse kidneys, and in mouse brains in vivo, 2′,3′-cAMP is metabolized to 2′-AMP and 3′-AMP; and these AMPs are subsequently converted to adenosine. In rat and mouse kidneys and mouse brains, injury releases 2′,3′-cAMP, 2′-AMP, and 3′-AMP into the extracellular compartment; and in humans, traumatic brain injury is associated with large increases in 2′,3′-cAMP, 2′-AMP, 3′-AMP, and adenosine in the cerebrospinal fluid. These findings motivate the *extracellular 2′,3′-cAMP-adenosine pathway hypothesis*: intracellular production of 2′,3′-cAMP → export of 2′,3′-cAMP → extracellular metabolism of 2′,3′-cAMP to 2′-AMP and 3′-AMP → extracellular metabolism of 2′-AMP and 3′-AMP to adenosine. Since 2′,3′-cAMP has been shown to activate mitochondrial permeability transition pores (mPTPs) leading to apoptosis and necrosis and since adenosine is generally tissue protective, the extracellular 2′,3′-cAMP-adenosine pathway may be a protective mechanism [i.e., removes 2′,3′-cAMP (an intracellular toxin) and forms adenosine (a tissue protectant)]. This appears to be the case in the brain where deficiency in CNPase (the enzyme that metabolizes 2′,3′-cAMP to 2-AMP) leads to increased susceptibility to brain injury and neurological diseases. Surprisingly, CNPase deficiency in the kidney actually protects against acute kidney injury, perhaps by preventing the formation of 2′-AMP (which turns out to be a renal vasoconstrictor) and by augmenting the mitophagy of damaged mitochondria. With regard to 2′,3′-cNMPs and their downstream metabolites, there is no doubt much more to be discovered.

Keywords
2′,3′-cAMP • 2′-AMP • 3′-AMP • Adenosine • CNPase • mPTP

1 Discovery of Nucleoside 2′,3′-Cyclic Monophosphates

Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) combines the efficiency of high- or ultra-performance LC to separate compounds with the power of MS/MS to differentiate compounds according to mass-to-charge ratios (m/z). In this regard, LC separates compounds by column retention time (RT) and MS/MS then selects a precursor ion with a specified m/z for further fragmentation to yield a product ion with a specified m/z that is selectively detected and quantified. This analytical procedure is known as selected reaction monitoring (SRM), and each monitored precursor/product ion pair is called a "transition."

While using LC-MS/MS to investigate the levels of adenosine 3′,5′-cyclic monophosphate (3′,5′-cAMP) in the renal venous outflow from isolated, perfused rat kidneys, Ren et al. (2009) observed a large chromatographic peak that was due to an SRM transition selected to detect specifically 3′,5′-cAMP (precursor ion, 330 m/z → product ion, 136 m/z). Yet the RT of the compound generating the signal was much shorter (2.9 min) than that for authentic 3′,5′-cAMP (6.3 min). The authors reasoned that although the signal could not have been due to 3′,5′-cAMP (because the RT did not match that of 3′,5′-cAMP), the signal must have arisen

from a compound similar in structure to 3′,5′-cAMP (because the SRM transition matched that of 3′,5′-cAMP). The authors hypothesized that the unknown substance might be adenosine 2′,3′-cyclic monophosphate (2′,3′-cAMP), which is structurally the simplest possible positional isomer of 3′,5′-cAMP that could give rise to the observed SRM transition. This hypothesis was confirmed by demonstrating that the unknown substance had: (1) the same RT as authentic 2′,3′-cAMP and (2) produced the same mass spectrum as authentic 2′,3′-cAMP at several different energies of collision-induced dissociation (Ren et al. 2009). Although an earlier report presented limited evidence for the existence of 2′,3′-cAMP in tobacco BY-2 cells (Richards et al. 2002), the study by Ren et al. (2009) was arguably the first unequivocal identification of 2′,3′-cAMP in any biological system and certainly the first detection of 2′,3′-cAMP in the animal kingdom.

The discovery of 2′,3′-cAMP in the rat kidney was rapidly followed by several other excellent publications that provided clear evidence for the existence in biological systems of not only 2′,3′-cAMP but also other nucleoside 2′,3′-cyclic monophosphates (2′,3′-cNMPs). Employing chromatography on porous graphitic carbon columns combined with quadrupole/time-of-flight (Q-TOF) mass spectrometry, Pabst and coworkers detected a signal consistent with 2′,3′-cAMP in tobacco plants (Pabst et al. 2010); and Van Damme and colleagues (2012), using solid-phase extraction on silica followed by LC-MS/MS, observed both 2′,3′-cAMP and guanosine 2′,3′-cyclic monophosphate (2′,3′-cGMP) in the rabbit pancreas and kidney and confirmed the identity of these 2′,3′-cNMPs using high mass-resolution TOF LC/MS. Burhenne and coworkers (2013) measured 2′,3′-cGMP, cytidine 2′,3′-cyclic monophosphate (2′,3′-cCMP), and uridine 2′,3′-cyclic monophosphate (2′,3′-cUMP) in mouse organs using LC-MS/MS (QTRAP®) and confirmed their identity using an extremely high mass-accuracy mass spectrometer (TripleTOF®). More recently, using LC-MS/MS, Bähre and Kaever (2014) detected 2′,3′-cAMP, 2′,3′-cGMP, 2′,3′-cGMP, and 2′,3′-cUMP in Hek293T cells (a human embryonic kidney cell line) and 2′,3′-cAMP, 2′,3′-cGMP, and 2′,3′-cGMP in HuT-78 cells (a human T cell lymphoma cell line). Another study by Van Damme and coworkers (2014) reported that 2′,3′-cAMP and 2′,3′-cGMP exist in plant tissue and increase fivefold upon wounding stress; these results were also generated using LC-MS/MS. Bordeleau et al. (2014) unequivocally identified (by HPLC, mass spectrometry, and NMR) 2′,3′-cCMP and 2′,3′-cUMP in cultures of *Pseudomonas fluorescens*. More recently, Jia et al. (2014) observed by LC-MS/MS the presence of 2′,3′-cAMP, 2′,3′-cGMP, 2′,3′-cCMP, and inosine 2′,3′-cyclic monophosphate (2′,3′-cIMP) in the rat kidney, lung, heart, spleen, liver, and brain. The accumulative evidence since 2009 establishes beyond any reasonable doubt that 2′,3′-cNMPs (2′,3′-cAMP, 2′,3′-cUMP, 2′,3′-cGMP, 2′,3′-cCMP, and 2′,3′-cIMP) are made by living systems and that these compounds with respect to the evolutionary time scale are very ancient biological molecules that are found in bacteria, plants, and animals.

2 Discovery of the 2′,3′-cAMP-Adenosine Pathway

2.1 The 3′,5′-cAMP-Adenosine Pathway Establishes a Precedent

The production of adenosine can occur through different mechanisms that are engaged by different stimuli to produce adenosine in different compartments and for different purposes. CD39 (ecto-nucleoside triphosphate diphosphohydrolase 1) working in tandem with CD73 (ecto-5′-nucleotidase) is a critically important "emergency" system for producing adenosine from ATP, ADP, or 5′-AMP. Importantly, inflammation and hypoxia activate the CD39/CD73 pathway, and this provides for the wholesale production of large quantities of adenosine to reduce inflammation and restore tissue perfusion (Eltzschig 2009, 2013; Eltzschig and Carmeliet 2011; Eltzschig et al. 2012). In contrast to the CD39/CD73 pathway, the "extracellular 3′,5′-cAMP-adenosine pathway" is a more nuanced system for producing adenosine in response to activation of adenylyl cyclase. This pathway involves the following sequential steps: intracellular synthesis of 3′,5′-cAMP from ATP by adenylyl cyclases; energy-dependent export of 3′,5′-cAMP from cells by cyclic nucleotide pumps [e.g., MRP4 (Cheng et al. 2010)]; conversion by ecto-3′,5′-cyclic nucleotide 3′-phosphodiesterases (yet to be identified) of extruded 3′,5′-cAMP to extracellular adenosine 5′-monophosphate (5′-AMP); metabolism by ecto-5′-nucleotidases (e.g., CD73 and tissue nonspecific alkaline phosphatase [TNAP]) of 5′-AMP to adenosine; and engagement of extracellular adenosine with adenosine receptors to expand, augment, or attenuate the effects of hormonal activation of adenylyl cyclase. The first explicit formulation of the extracellular 3′,5′-cAMP-adenosine pathway was postulated in 1991 as part of a transmembrane negative feedback mechanism to control renin release from juxtaglomerular cells (Jackson 1991). Since then, a large body of evidence has accumulated supporting the view that 3′,5′-cAMP can serve as a "third messenger" as part of the extracellular 3′,5′-cAMP-adenosine pathway. For example, studies show that the extracellular 3′,5′-cAMP-adenosine pathway exists in the: (1) kidneys (Mi et al. 1994; Mi and Jackson 1995, 1998; Dubey et al. 1996, 1998, 2000a, b, 2001, 2010; Jackson et al. 1997, 2003, 2006, 2007b; Jackson and Mi 2000, 2008; Kuzhikandathil et al. 2011), (2) pial microvessels (Hong et al. 1999), (3) skeletal muscle (Chiavegatti et al. 2008; Duarte et al. 2012), (4) gastrointestinal tract (Giron et al. 2008), (5) fat (Müller et al. 2008), (6) neuronal cells (Do et al. 2007), (7) sperm (Osycka-Salut et al. 2014), and (8) immune cells (Sciaraffia et al. 2014). In addition to contributing to a local signaling network, the extracellular 3′,5′-cAMP-adenosine may also function as part of a circulating hormonal system by which the liver can alter the function of distal organs via the release of an adenosine precursor (3′,5′-cAMP) that is delivered to tissues by the circulation and subsequently metabolized to adenosine within the target organ (Jackson et al. 2007a). Although the half-life of adenosine in blood is too short (in humans only seconds) for adenosine to serve as a circulating hormone, 3′,5′-cAMP is stable in blood and thus can be released distally and converted locally to adenosine.

2.2 Evidence for the 2′,3′-cAMP-Adenosine Pathway

With the extracellular 3′,5′-cAMP-adenosine pathway serving as a prototype, after the discovery of 2′,3′-cAMP in a mammalian system, Jackson and coworkers (2009) postulated the existence of an "extracellular 2′,3′-cAMP-adenosine pathway" (intracellular production of 2′,3′-cAMP → export of 2′,3′-cAMP → extracellular metabolism of 2′,3′-cAMP to 2′-AMP and 3′-AMP → extracellular metabolism of 2′-AMP and 3′-AMP to adenosine). Several lines of reasoning converge to support this hypothesis.

The first line of reasoning supporting the abovementioned pathway is that 2′,3′-cAMP is produced intracellularly. Degradation of intracellular RNA is mediated by intracellular ribonucleases (RNases) that facilitate the hydrolysis of the P–$O^{5'}$ bond of RNA by transphosphorylation of RNA to yield 2′,3′-cNMPs followed by hydrolysis of 2′,3′-cNMPs to 3′-nucleoside monophosphates (3′-NMPs) (Wilusz et al. 2001). Importantly, ^{31}P NMR spectroscopy (Thompson et al. 1994) demonstrates that only a small fraction (0.1%) of 2′,3′-cNMPs formed by transphosphorylation of RNA is hydrolyzed before dissociating from RNases. Instead, apparently RNases release mostly intact 2′,3′-cNMPs. Thus, 2′,3′-cNMPs (including 2′,3′-cAMP) are likely formed from nucleotide bases in RNA by RNase-catalyzed transphosphorylation reactions (Thompson et al. 1994). Recent studies by Sokurenko et al. (2015) support this concept, and studies by Gu et al. (2013) using kinetic isotope effect analysis, molecular dynamics simulations, and quantum mechanical calculations provide a detailed molecular mechanism responsible for the biosynthesis of 2′,3′-cNMPs. Because mRNA has a large quantity of adenosine monophosphates in the poly-A tail (Alberts et al. 1989), mRNA turnover has the potential to generate particularly large amounts of 2′,3′-cAMP (but also other 2′,3′-cNMPs as well).

The second line of evidence supporting the extracellular 2′,3′-cAMP-adenosine pathway is that nucleotide transporters, such as MRP4 and MRP5, rapidly and actively export a diverse array of linear and cyclic nucleotides into the extracellular compartment (Kruh et al. 2001; van Aubel et al. 2002; Deeley et al. 2006; Borst et al. 2007). Although not yet tested, it is likely that at least a subset of nucleotide transporters would export 2′,3′-cAMP. Indeed, release of endogenous 2′,3′-cAMP into the renal circulation of isolated, perfused rat and mouse kidneys (Jackson et al. 2009, 2011b; Ren et al. 2009) indicates that 2′,3′-cAMP does reach the extracellular compartment.

The third line of support for the extracellular 2′,3′-cAMP-adenosine pathway is that there are a number of enzymes that could conceivably serve as ecto-2′,3′-cyclic nucleotide 3′-phosphodiesterases, ecto-2′,3′-cyclic nucleotide 2′-phosphodiesterases, ecto-2′-nucleotidases and ecto-3′-nucleotidases to hydrolyze extracellular 2′,3′-cAMP to 2′-AMP, extracellular 2′,3′-cAMP to 3′-AMP, extracellular 2′-AMP to adenosine, and extracellular 3′-AMP to adenosine, respectively. A prime candidate for ecto-2′,3′-cyclic nucleotide 3′-phosphodiesterase activity in vivo is 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) which is a protein that in vitro can metabolize 2′,3′-cAMP to 2′-AMP (Vogel and Thompson 1988;

Sprinkle 1989; Thompson 1992). Also, some RNases (e.g., ptRNase 1) are secreted by cells and can hydrolyze 2′,3′-cAMP to 3′-AMP (Sorrentino and Libonati 1997; Sorrentino 1998); and Rao et al. (2010) report that six different phosphodiesterases containing three different families of hydrolytic domains can generate 3′-AMP from 2′,3′-cAMP. Consistent with the existence of ecto-2′,3′-cyclic nucleotide 2′-phosphodiesterases and ecto-2′,3′-cyclic nucleotide 3′-phosphodiesterases are the findings that 3′-AMP is present in the rat spleen, kidney, liver, heart, and brain (Bushfield et al. 1990; Fujimori and Pan-Hou 1998; Fujimori et al. 1998; Miyamoto et al. 2008) and that 2′-AMP and 3′-AMP are present in the human cerebrospinal spinal fluid (CSF), and their concentrations correlate with the concentrations of 2′,3′-cAMP (Verrier et al. 2012). Finally, there are ecto-nucleotidases that can function to process extracellular 2′-AMP and 3′-AMP to adenosine. For example, studies by Ohkubo et al. (2000) show that in NG108-15 cells, TNAP can release orthophosphate from either 2′-AMP or 3′-AMP. Moreover, TNAP metabolizes 2′-AMP and 3′-AMP to adenosine both in isolated, perfused rat kidneys and in rat kidneys in vivo (Jackson, E.K., unpublished observations). Also, levels of adenosine in human CSF correlate with levels of 2′,3′-cAMP, 2′-AMP, and 3′-AMP (Verrier et al. 2012), a finding consistent with the metabolism of these compounds to adenosine in the extracellular compartment.

As an initial test for whether an extracellular 2′,3′-cAMP-adenosine pathway exists, Jackson et al. (2009) infused 2′,3′-cAMP (30 μmol/L) into the renal arteries of isolated, perfused rat kidneys and measured renal venous levels of potential metabolites. Infusions of 2′,3′-cAMP increased the mean renal venous secretions of 3′-AMP, 2′-AMP, and adenosine by 3,400-fold, 26,000-fold, and 53-fold, respectively. Similar to rat kidneys, intrarenal artery infusions of 2′,3′-cAMP into isolated, perfused mouse kidneys also increased renal venous secretions of 2′-AMP, 3′-AMP, and adenosine (Jackson et al. 2011b). In isolated, perfused rat kidneys, arterial infusions of 2′-AMP and 3′-AMP augmented renal venous secretion of adenosine similar to that achieved by 5′-AMP. Since 5′-AMP is viewed as the main precursor for the production of endogenous adenosine, the fact that 2′-AMP and 3′-AMP can be converted to adenosine in intact kidneys with an efficiency similar to 5′-AMP strongly supports the extracellular 2′,3′-cAMP-adenosine pathway hypothesis. Importantly, renal artery infusions of 2′,3′-cAMP in anesthetized rats (Jackson et al. 2009) increased urinary excretion of 2′-AMP, 3′-AMP, and adenosine, and intrarenal artery infusions of 2′-AMP and 3′-AMP increased urinary excretion of adenosine as efficiently as did 5′-AMP. These findings support the conclusion that the extracellular 2′,3′-cAMP-adenosine pathway may exist in vivo.

As discussed above, 2′,3′-cAMP is most likely a product of RNA degradation, and several studies in intact tissues indicate that energy depletion can activate the degradation of RNA (Akahane et al. 2001a, b; Almeida et al. 2004). Therefore, to test the concept that endogenous 2′,3′-cAMP can be converted to 2′-AMP, 3′-AMP, and adenosine, Jackson et al. assessed the effects of energy depletion with metabolic inhibitors (iodoacetate plus 2,4-dinitrophenol) on the renal venous secretion of 2′,3′-cAMP, 2′-AMP, 3′-AMP, and adenosine. In isolated, perfused rat kidneys, metabolic inhibitors augmented the mean renal venous secretion of 2′,3′-cAMP,

3′-AMP, 2′-AMP, and adenosine by 29-fold, 16-fold, 10-fold, and 4.2-fold, respectively (Jackson et al. 2009). Similar findings were observed in isolated, perfused mouse kidneys (Jackson et al. 2011b). To further test the notion that 2′,3′-cAMP in intact tissues derives from RNA breakdown, isolated, perfused rat kidneys were treated with rapamycin, an agent that stimulates RNA turnover by engaging the mTOR pathway (Banholzer et al. 1997; Hashemolhosseini et al. 1998; Albig and Decker 2001). Indeed, rapamycin caused a time-related increase in renal venous 2′,3′-cAMP secretion but inhibited the renal secretion of 3′,5′-cAMP (Ren et al. 2009).

Unlike 3′,5′-cAMP, 2′,3′-cAMP does not function as a circulating adenosine precursor. When added to either whole blood or plasma, 2′,3′-cAMP, 2′-AMP, and 3′-AMP disappear in less than 1 min (Jackson, E.K., unpublished observation). Moreover, in isolated, perfused mouse kidneys, approximately 95% of arterial 2′,3′-cAMP is removed from the vascular compartment during a single pass through the kidney (Jackson et al. 2011b), suggesting that with regard to the extracellular 2′,3′-cAMP-adenosine pathway, what happens locally stays local.

The extracellular 2′,3′-cAMP-adenosine pathway may exist in many cells, tissues, and organs. In support of this conclusion, preglomerular vascular smooth muscle cells (Jackson et al. 2010), preglomerular vascular endothelial cells (Jackson and Gillespie 2012), glomerular mesangial cells (Jackson et al. 2010), renal epithelial cells all along the nephron (Jackson and Gillespie 2012, 2013a), aortic vascular smooth muscle cells (Jackson et al. 2011c), coronary artery vascular smooth muscle cells (Jackson et al. 2011c), microglia (Verrier et al. 2011), astrocytes (Verrier et al. 2011), oligodendrocytes (Verrier et al. 2013), neurons (Verrier et al. 2013), Schwann cells (Verrier et al. 2015), and intact brain in vivo (Verrier et al. 2012) can metabolize exogenous 2′,3′-cAMP, 2′-AMP, and 3′-AMP to downstream purines.

It is important to note that 2′,3′-cAMP elutes faster on reverse phase chromatographic columns compared to 3′,5′-cAMP, indicating that 2′,3′-cAMP is even more hydrophilic than is 3′,5′-cAMP. This implies that, like exogenous 3′,5′-cAMP, exogenous 2′,3′-cAMP would have little ability to pass through cell membranes. It is likely, therefore, that the aforementioned experiments with exogenous 2′,3′-cAMP imply the existence of an extracellular 2′,3′-cAMP-adenosine pathway. However, in addition to the extracellular 2′,3′-cAMP-adenosine pathway, other configurations of the 2′,3′-cAMP-adenosine pathway are certainly possible (e.g., the transcellular and intracellular 2′,3′-cAMP-adenosine pathways, see Fig. 1). Indeed, it is possible that some of the extracellularly detected 2′-AMP, 3′-AMP, and adenosine generated by experiments using energy depletion or rapamycin derived from intracellular conversion of 2′,3′-cAMP to 2′-AMP and 3′-AMP or intracellular metabolism of these AMPs to adenosine followed by export of the produced AMPs or adenosine. The same can be said for the 3′,5′-cAMP-adenosine pathway, i.e., in addition to the extracellular 3′,5′-cAMP-adenosine pathway, transcellular and intracellular modes may exist.

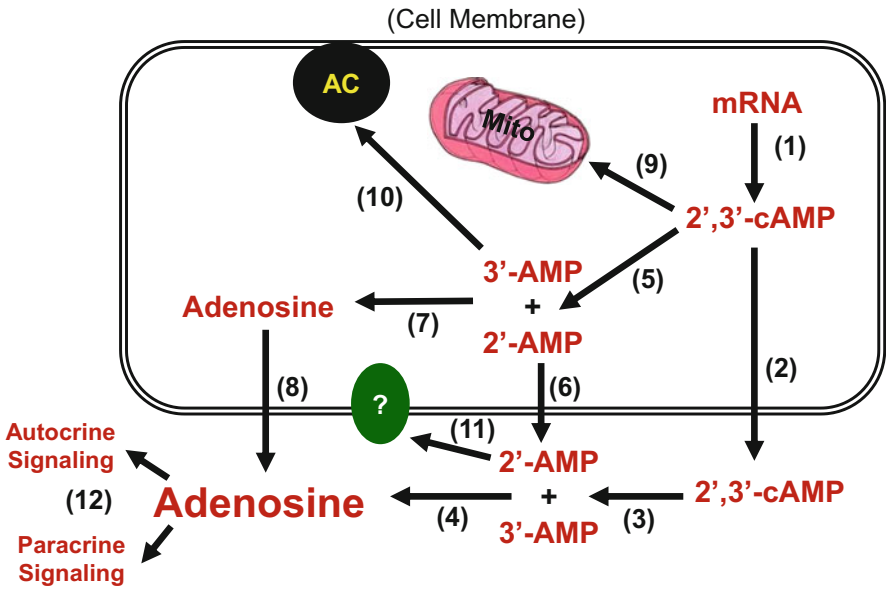

Fig. 1 Depiction of the extracellular, intracellular, and transcellular 2′,3′-cAMP-adenosine pathways. *1*: Production of 2′,3′-cAMP from RNase-mediated degradation of mRNA. *2*: Active transport of 2′,3′-cAMP into the extracellular compartment by cyclic nucleotide pumps (e.g., MRP4 and MRP5). *3*: Metabolism of extracellular 2′,3′-cAMP to 2′-AMP and 3′-AMP by ecto-2′,3′-cyclic nucleotide 3′-phosphodiesterses (e.g., extracellular CNPase) and ecto-2′,3′-cyclic nucleotide 2′-phosphodiesterses (e.g., extracellular RNases), respectively. *4*: Metabolism of extracellular 2′-AMP and 3′-AMP to adenosine by ecto-nucleotidases (e.g., tissue nonspecific alkaline phosphatase). *5*: Metabolism of intracellular 2′,3′-cAMP to 2′-AMP and 3′-AMP by endo-2′,3′-cyclic nucleotide 3′-phosphodiesterses (e.g., intracellular CNPase) and endo-2′,3′-cyclic nucleotide 2′-phosphodiesterses (e.g., intracellular RNases), respectively. *6*: Active transport of 2′-AMP/3′-AMP into the extracellular compartment by nucleotide transporters. *7*: Dephosphorylation of 2′-AMP and 3′-AMP to adenosine by endo-nucleotidases. *8*: Efflux of adenosine to the extracellular compartment mediated by equilibrative nucleoside transporters (e.g., ENT1 and ENT2). *9*: Opening of mitochondrial permeability transition pores. *10*: P-site inhibition of adenylyl cyclase (AC). *11*: Activation of unknown receptor leading to renal vasoconstriction. *12*: Autocrine and paracrine signaling by adenosine via cell-surface adenosine receptors. Extracellular 2′,3′-cAMP-adenosine pathway = 1 → 2 → 3 → 4; intracellular 2′,3′-cAMP-adenosine pathway = 1 → 5 → 7 → 8; transcellular 2′,3′-cAMP-adenosine pathway = 1 → 5 → 6 → 4

2.3 Enzymes Involved in the 2′,3′-cAMP-Adenosine Pathway

CNPase is the most abundant protein in the non-compact CNS myelin and is the third most abundant protein overall in the CNS myelin (Raasakka and Kursula 2014). Even so, the physiological role for CNPase remained an enigma from the time of its discovery in the bovine spleen in 1955 (Whitfeld et al. 1955) until the discovery of 2′,3′-cAMP in rat kidneys in 2009 (Ren et al. 2009). Because, prior to 2009, 2′,3′-cAMP was not known to exist in biological systems, the ability of

CNPase to convert 2′,3′-cAMP to 2′-AMP in vitro was considered an epiphenomenon (Vogel and Thompson 1988; Thompson 1992; Schmidt 1999). Due to the discovery of 2′,3′-cAMP production by the rat kidney, the physiological role of CNPase is now viewed in a new light (Raasakka and Kursula 2014).

There exist two CNPase mRNAs that are generated by alternative splicing of an RNA transcript generated from a single gene (Vogel and Thompson 1988; Sprinkle 1989; Thompson 1992; Schmidt 1999). One splice variant codes for CNPase I isoform of the enzyme, and the other variant has two potential translation initiation sites and can give rise to both isoforms of the enzyme. Compared to CNPase I, CNPase II has an additional 20 amino acids on the N-terminus that serves as a mitochondrial-targeting sequence which is removed after import of CNPase II into the intermembrane space (Raasakka and Kursula 2014). CNPase is modified by isoprenylation at the C-terminus and has several closely spaced hydrophobic domains. These molecular features cause the attachment of CNPase to membranes.

Studies published in 2012 (Verrier et al. 2012) were the first to reveal an important physiological role for the catalytic activity of CNPase. In the brain, this enzyme metabolizes endogenous 2′,3′-cAMP to 2′-AMP and thereby reduces levels of 2′,3′-cAMP and increases the biosynthesis of adenosine (i.e., CNPase contributes importantly to the brain extracellular 2′,3′-cAMP-adenosine pathway). Using microdialysis probes both to deliver 2′,3′-cAMP to the extracellular compartment of the mouse brain and to analyze the conversion of 2′,3′-cAMP to purines in that compartment, these investigators showed that the brain metabolizes exogenous (extracellular) 2′,3′-cAMP to 2′-AMP and that the conversion of exogenous (extracellular) 2′,3′-cAMP to 2′-AMP is impaired in vivo in brains from CNPase knockout mice. This investigative team also showed that in wild-type mice, traumatic brain injury (TBI, controlled cortical impact model) activated the brain 2′,3′-cAMP-adenosine pathway [i.e., TBI increased brain interstitial levels of 2′,3′-cAMP, 2′-AMP, 3′-AMP, adenosine, and inosine (adenosine metabolite)]. In CNPase knockout mice compared to wild-type mice, TBI induced higher levels of interstitial 2′,3′-cAMP, yet lower levels of 2′-AMP, adenosine, and inosine, indicating that deficiency of CNPase leads to impairment of the 2′,3′-cAMP-adenosine pathway (Verrier et al. 2012). This study was the first demonstration that the enzymatic activity of CNPase plays an important role in a biochemical pathway. Moreover, histological analysis suggested that TBI was associated with greater neuronal injury in the hippocampus of CNPase knockout mice compared with wild-type mice. Because adenosine is neuroprotective (Kochanek et al. 2013b) and 2′,3′-cAMP opens mitochondrial permeability transition pores (mPTPs) (Azarashvili et al. 2009, 2010) leading to apoptosis and necrosis, the metabolism of endogenous 2′,3′-cAMP to 2′-AMP by CNPase may rid the brain of an intracellular neurotoxin (2′,3′-cAMP) while providing a neuroprotectant (adenosine). Indeed, in the CSF from TBI patients, 2′,3′-cAMP was significantly increased in the initial 12 h after injury and strongly correlated with CSF levels of 2′-AMP, 3′-AMP, adenosine, and inosine. Taken together, these findings suggest that: (1) the 2′,3′-cAMP-adenosine pathway exists in vivo in brains of both mice and humans, (2) the brain CNPase is involved in the metabolism of endogenously generated

2′,3′-cAMP to 2′-AMP, and (3) the 2′,3′-cAMP-adenosine pathway and CNPase are important with regard to protecting the brain from injury. This latter point is underscored by the observations that CNPase knockout mice have a number of brain histological and functional abnormalities including: (1) axonal degeneration beginning early in life (Lappe-Siefke et al. 2003; Edgar et al. 2009); (2) enhanced astrogliosis, microgliosis, axon degeneration, and defects in working memory following mild brain injury (Wieser et al. 2013); and (3) an aging-associated psychiatric disease described as "catatonia-depression" syndrome (Hagemeyer et al. 2012).

With regard to the brain extracellular 2′,3′-cAMP-adenosine pathway, different CNS cells most likely contribute differentially to the pathway sequence. For example, although astrocytes, microglia, oligodendrocytes, and neurons can metabolize 2′,3′-cAMP to 2′-AMP, oligodendrocytes excel at this (Verrier et al. 2013). Likely, this is because oligodendrocytes are enriched in CNPase. Indeed, in oligodendrocytes from CNPase knockout mice, the metabolism of 2′,3′-cAMP to 2′-AMP is impaired (Verrier et al. 2013). On the other hand, microglia are the best at converting 2′-AMP to adenosine (Verrier et al. 2011). Although brain injury increases extracellular 2′,3′-cAMP levels, the major source of 2′,3′-cAMP has not yet been identified. Therefore, in the brain, a collaboration among cell types may be required to constitute a complete 2′,3′-cAMP-adenosine pathway.

Although the general view is that CNPase is primarily a brain protein, it is conceivable that this enzyme may also be important in mediating the 2′,3′-cAMP-adenosine pathway in other tissues and organ systems. Because adenosine is important in modulating renal function (Vallon et al. 2006), Jackson and coworkers recently evaluated the role of CNPase in the kidney (Jackson et al. 2014b). Importantly, western blotting and real-time PCR demonstrated robust expression of CNPase in rat glomerular mesangial, preglomerular vascular smooth muscle and endothelial, proximal tubular, thick ascending limb, and collecting duct cells (Jackson et al. 2014b). Moreover, real-time PCR detected expression of CNPase mRNA in human glomerular mesangial, proximal tubular, and vascular smooth muscle cells; and the level of expression of CNPase mRNA in these human cells was greater than the expression of mRNA for phosphodiesterase 4 (a major enzyme for the metabolism of 3′,5′-cAMP) (Jackson et al. 2014b). Intrarenal artery administration of 2′,3′-cAMP into isolated, perfused mouse kidneys increased renal venous 2′-AMP, and this response was diminished by 63% in kidneys from CNPase knockout mice, whereas the conversion of 3′,5′-cAMP to 5′-AMP was similar in kidneys from wild-type versus CNPase knockout mice. In normal mouse kidneys, energy depletion with metabolic poisons increased kidney tissue levels of adenosine and its metabolites (inosine, hypoxanthine, xanthine, and uric acid) without accumulation of 2′,3′-cAMP, whereas in CNPase-deficient kidneys, energy depletion increased kidney tissue levels of 2′,3′-cAMP but failed to increase adenosine and its metabolites. Taken together, these findings indicate that the kidneys express CNPase, and renal CNPase mediates in part the renal 2′,3′-cAMP-adenosine pathway.

Overexpressing CNPase in cells results in increased metabolism of exogenous 2′,3′-cAMP to 2′-AMP (Jackson et al. 2014b). Since exogenous 2′,3′-cAMP likely does not penetrate into cells, this implies that CNPase can exist external to the cell membrane. Because CNPase has several stretches of hydrophobic amino acids and undergoes isoprenylation and palmitoylation (Thompson 1992), CNPase associates tightly with membranes, including the intracellular aspect of the cell membrane (Thompson 1992) as well as with mitochondrial membranes (Azarashvili et al. 2009). If CNPase can be released by cells, then likely it would also associate with the extracellular aspect of cell membranes. As reviewed by Arnoys and Wang (Arnoys and Wang 2007), many intracellular proteins exhibit dual localization (intracellular and extracellular) due to mechanisms such as membrane blebbing leading to the release of proteins accumulated under specific regions of the plasma membrane or exosomal release. Of note is the fact that CNPase is localized to lipid rafts (Hinman et al. 2008) and accumulates in exosomes (Fruhbeis et al. 2013), so dual localization would be expected. Indeed, studies show that in humans, CNPase occurs in the cerebrospinal fluid (Vogel and Thompson 1988), suggesting that this protein does escape the intracellular compartment. It is likely, then, that CNPase contributes to the extracellular, intracellular, and transcellular 2′,3′-cAMP-adenosine pathways (Fig. 1).

Because of the potential importance of CNPase in the 2′,3′-cAMP-adenosine pathway, a potent and selective inhibitor of CNPase would be a valuable pharmacological agent for investigating the physiological roles of 2′,3′-cAMP metabolism in organ systems. Although currently there are no selective, potent inhibitors of CNPase, previous studies in rat preglomerular vascular smooth muscle cells (Jackson et al. 2010), mouse microglia and astrocytes (Verrier et al. 2011), and rat Schwann cells (Verrier et al. 2015) show that 1,3-dipropyl-8-(p-sulfophenyl) xanthine (DPSPX) partially blocks the conversion of 2′,3′-cAMP to 2′-AMP (but not 3′-AMP). Moreover, 1 and 10 mmol/L of DPSPX inhibits recombinant human CNPase by 35% and 75%, respectively (Verrier et al. 2015). Therefore, DPSPX may provide a structural platform for developing more potent and selective CNPase inhibitors.

Although CNPase can metabolize 2′,3′-cAMP to 2′-AMP, it does not metabolize 2′,3′-cAMP to 3′-AMP. Interestingly, in some experimental paradigms, 2′,3′-cAMP is metabolized mostly to 2′-AMP, and in other settings a balance of 2′-AMP and 3′-AMP is produced (Jackson, E. K., unpublished observation). What enzymes metabolize 2′,3′-cAMP to 3′-AMP? Studies by Rao et al. have begun to address this question. These investigators have identified six phosphohydrolases from microorganisms that metabolize 2′,3′-cAMP mostly to 3′-AMP (Rao et al. 2010). The near exclusive production of 2′,3′-cAMP to 3′-AMP is likely related to the lower activation energy of the P–$O^{2'}$ bond of 2′,3′-cAMP. The presence of 2′,3′-cyclic nucleotide 2′-phosphodiesterases in prokaryotes suggests that similar enzymes exist in the animal kingdom and can serve as ecto- and/or endo-2′,3′-cyclic nucleotide 2′-phosphodiesterases that metabolize extracellular and intracellular 2′,3′-cAMP to 3′-AMP.

Currently, very little is known regarding the identity of either extracellular or intracellular 2′- or 3′-nucleotidases that metabolize extracellular and intracellular 2′-AMP and 3′-AMP to adenosine. What is known is that CD73 is not involved in the 2′,3′-cAMP-adenosine pathway. This conclusion is based on the observations that pharmacological inhibition of CD73 does not alter the metabolism of extracellular 2′-AMP or 3′-AMP to adenosine in any cell types so far tested (Jackson et al. 2010, 2011c; Verrier et al. 2011), and the metabolism of extracellular 2′,3′-cAMP to adenosine is similar in kidneys from CD73 knockout versus wild-type mice (Jackson et al. 2011b). However, in isolated, perfused rat kidneys or rat kidneys in vivo, the metabolism of 2′-AMP to 3′-AMP is inhibited by L-p-bromotetramisole (a TNAP inhibitor) suggesting that TNAP is the major enzyme in mammals responsible for ecto-2′-nucleotidase and ecto-3′-nucleotidase activity (Jackson, E.K., unpublished observation).

3 Pharmacology of 2′,3′-cAMP, 2′-AMP, and 3′AMP

Before the discovery of the existence of 2′,3′-cAMP in biological systems in 2009, there were only a small number of published studies on the pharmacological effects of 2′-AMP, 3′-AMP, and 2′,3′-cAMP; and many of these early studies were contradictory. For example, in cats, intravenous boluses of 2′-AMP, 3′-AMP, and 2′,3′-cAMP were reported to cause a delayed reduction in arterial blood pressure (reaching a maximum in approximately 5 min followed by a recovery, which took 20–30 min) that was accompanied by baroreceptor-induced tachycardia; and intra-arterial delivery of 2′-AMP, 3′-AMP, and 2′,3′-cAMP was observed to induce vasodilation of the guinea pig hind limb preparation (Denatale et al. 1963). In contrast, 2′,3′-cAMP was found not to vasodilate the isolated, perfused canine coronary artery preparation (Nakane and Chiba 1993).

Many of the reports regarding the biochemical and cellular effects of 2′-AMP, 3′-AMP, and 2′,3′-cAMP were negative. 2′,3′-cAMP was observed to have little or no effect on protein kinase activity (Amrhein and Filner 1973), hepatic glucose production (Garrison and Haynes 1975), neural differentiation of cultured amphibian cells (Wahn et al. 1975), pacemaker cells in the frog heart (Hartzell 1979), or the formation of granulocyte-macrophage colonies in cultures of the mouse bone marrow (Fleming and McNeill 1976). Moreover, 3′-AMP did not attenuate growth of mastocytoma P-815 cells (Ichikawa et al. 1980).

However, there were reports of positive findings. 2′,3′-cAMP was shown to inhibit leucine incorporation in a mouse lymphoma cell line (Fuhr and Stidham 1980), to reduce neurotransmission in the rat vas deferens (Willemot and Paton 1981; Fiszman and Stefano 1984), and to affect damselfish motile iridophores (Oshima et al. 1986). Also, 2′-AMP was reported to inhibit evoked potentials in the rat hippocampal slice preparation (Lee et al. 1981), and 3′-AMP was found to be an effective P-site inhibitor of adenylyl cyclase (Johnson et al. 1989; Bushfield et al. 1990). Importantly, the presence of 3′-AMP in tissues has been confirmed (Fujimori and Pan-Hou 1998; Fujimori et al. 1998; Miyamoto et al. 2008), which

suggests that P-site inhibition by 3′-AMP may be physiologically relevant. The ability of 3′-AMP to inhibit adenylyl cyclase implies that generation of 3′-AMP by the intracellular metabolism of 2′,3′-cAMP to 3′-AMP could be an important mechanism by which intracellular 2′,3′-cAMP regulates cell signaling.

Until 2009, the world's literature on 2′,3′-cAMP, 2′-AMP, and 3′-AMP was scant, incomplete, inconsistent, and without apparent significance. That changed in 2009 with three discoveries: (1) 2′,3-cAMP is formed in mammalian systems (Ren et al. 2009); (2) 2′,3′-cAMP can be converted to adenosine (Jackson et al. 2009); and (3) 2′,3′-cAMP regulates calcium-dependent permeability transition in rat brain mitochondria (Azarashvili et al. 2009, 2010). These discoveries suggested a novel mechanism for 2′,3′-cAMP in physiology.

Motivated by these events, beginning in 2010, Jackson and coworkers investigated the pharmacology of 2′,3′-cAMP, 2′-AMP, and 3′-AMP in several important biological systems. This research team found that in rat preglomerular vascular smooth muscle cells and glomerular mesangial cells and in human aortic and coronary vascular smooth muscle cells, 2′,3′-cAMP, 2′-AMP, and 3′-AMP potently inhibited cell proliferation (Jackson et al. 2010, 2011a, c). They also observed that these inhibitory effects on cell proliferation were mediated by the metabolism of 2′,3′-cAMP, 2′-AMP, and 3′-AMP to adenosine, which inhibited growth of these cell types via engaging A_{2B} receptors (Jackson et al. 2010, 2011a, c). Unlike vascular smooth muscle and glomerular mesangial cells, 2′,3′-cAMP, 2′-AMP, and 3′-AMP stimulated the proliferation of vascular endothelial cells and renal epithelial cells, and again the mechanism involved adenosine and A_{2B} receptors (Jackson and Gillespie 2012). Excess proliferation of vascular smooth muscle cells and deficient growth of vascular endothelial cells can lead to vascular disease; and excess growth of glomerular mesangial cell and reduced proliferation of renal epithelial cells can contribute to renal dysfunction. Taken together, the pharmacology of 2′,3′-cAMP, 2′-AMP, and 3′-AMP in these cell types suggests that the 2′,3′-cAMP-adenosine pathway may protect the vasculature.

Although clearly 2′,3′-cAMP, 2′-AMP, and 3′-AMP are metabolized to adenosine in vitro, whether this occurs sufficiently rapid for these purines to exert acute effects via adenosine receptors in vivo was unknown. To address this question, Jackson and Mi (2013) compared the cardiovascular and renal effects of 2′,3′-cAMP, 2′-AMP, 3′-AMP, 3′,5′-cAMP, 5′-AMP, and adenosine in vivo in the rat. 2′,3′-cAMP, 2′-AMP, and 3′-AMP dose dependently and profoundly reduced arterial blood pressure, heart rate, total peripheral resistance, and mesenteric vascular resistance with an efficacy and potency similar to adenosine and 5′-AMP. Moreover, these effects of 2′,3′-cAMP, 2′-AMP, and 3′-AMP were decreased by blockade of adenosine receptors. Also, 2′,3′-cAMP increased urinary excretion rates of 2′-AMP, 3′-AMP, and adenosine. These results demonstrated that in vivo 2′,3′-cAMP, 2′-AMP, and 3′-AMP can be considered adenosine "prodrugs" and may have clinical utility for the same indications as adenosine, for example, terminating supraventricular tachycardias and as an adjunct to thallium-201 myocardial perfusion scintigraphy in patients unable to exercise adequately. 2′,3′-cAMP may also have benefits in surgery. Studies in 2004 showed that

adenosine administered into the peritoneal cavity attenuated the formation of postsurgical adhesions, but full efficacy required multiple dosing over 3 days (Jackson 2004). However, a recent study demonstrated that administration of a single dose of 2′,3′-cAMP into the peritoneal cavity dramatically reduced the formation of postsurgical adhesions in mice, likely by serving as an adenosine prodrug to increase the exposure time of the damaged tissue to adenosine (Forman et al. 2014).

4 Physiological Roles of the 2′,3′-cAMP-Adenosine Pathway

4.1 Neuroprotection

Tissue injury initiates RNA degradation (Akahane et al. 2001a, b; Almeida et al. 2004; Catts et al. 2005; Chevyreva et al. 2008). Therefore, brain trauma and neurodegenerative diseases could activate the 2′,3′-cAMP-adenosine pathway. As mentioned above, studies by Jackson and colleagues show that traumatic injury to the brain does indeed increase brain interstitial levels of 2′,3′-cAMP, 2′-AMP, 3′-AMP, and adenosine in mice and increases CSF levels of these purines in humans (Verrier et al. 2012). A key study by Azarashvili et al. demonstrates that 2′,3′-cAMP facilitates the opening of mPTPs in brain mitochondria (Azarashvili et al. 2009), and it is well accepted that opening of mPTPs can lead to apoptosis (Kroemer et al. 2007). Thus, the fact that RNA breakdown is one of the earliest events associated with apoptosis (Del Prete et al. 2002) could be explained by the following sequence of events: injury → RNA degradation → 2′,3′-cAMP formation → opening of mPTPs → apoptosis. Since the 2′,3′-cAMP-adenosine pathway involves the cellular export and metabolism of 2′,3′-cAMP, this pathway should be neuroprotective. Additionally, there is now a large body of evidence indicating that adenosine is neuroprotective (Marangos et al. 1990; Fern et al. 1994; Robertson et al. 1999; Hendrich et al. 2001; Kochanek and Jackson 2001; Phillis and Goshgarian 2001; Stone 2002; Boison 2007; Chen et al. 2007; Haselkorn et al. 2010; Thauerer et al. 2012; Kochanek et al. 2013a, b). Therefore, not only does the 2′,3′-cAMP-adenosine eliminate a neurotoxin (2′,3′-cAMP), it also promotes the biosynthesis of a neuroprotectant (adenosine). Along these lines, recent studies by Newell et al. show that 2′,3′-cAMP, 3′-AMP, and 2′-AMP inhibit the release of TNF-α (proinflammatory cytokine) and CXCL10 (proinflammatory chemokine) by primary murine microglia via production of adenosine leading to activation of A_{2A} receptors (Newell et al. 2015). As mentioned earlier, knockout of CNPase impairs the brain 2′,3′-cAMP-adenosine pathway and: (1) worsens histological outcome in TBI (Verrier et al. 2012); (2) causes axonal degeneration beginning early in life (Lappe-Siefke et al. 2003; Edgar et al. 2009); (3) enhances mild brain injury-induced astrogliosis, microgliosis, axon degeneration, and defects in working memory (Wieser et al. 2013); and (4) promotes aging-associated psychiatric diseases (Hagemeyer et al. 2012). Enhancing the brain 2′,3′-cAMP-adenosine pathway by augmenting the export of 2′,3′-cAMP or increasing its

metabolism to adenosine may offer effective treatments for brain injuries and diseases.

4.2 Renoprotection

It is well known that extracellular adenosine is renoprotective. Studies by Okusa and coworkers demonstrate that adenosine, via A_{2A} receptors, protects the kidney from ischemia/reperfusion injury (and other forms of injury) primarily by anti-inflammatory actions mediated by A_{2A} receptors (Okusa et al. 1999, 2000, 2001; Okusa 2002; Day et al. 2005). Studies show that both A_{2B} (Grenz et al. 2008) and A_1 receptors (Lee and Emala 2000, 2002; Lee et al. 2007; Kim et al. 2009) also protect the kidney from ischemia/reperfusion injury. Active transport of intracellular 2′,3′-cAMP to the cell surface followed by extracellular metabolism of 2′,3′-cAMP to corresponding AMPs and to adenosine should maintain low intracellular levels of 2′,3′-cAMP and high extracellular levels of adenosine. Thus, just as in the case of the brain, one would predict that the 2′,3′-cAMP-adenosine pathway should protect the kidney from injury. In a surprising twist, this prediction turns out to be entirely incorrect.

To determine whether the 2′,3′-cAMP-adenosine pathway is renoprotective, Jackson et al. (2015) investigated renal outcomes following acute kidney injury (AKI) in CNPase knockout (CNPase−/−) versus wild-type (CNPase +/+) mice. AKI was induced by bilateral renal ischemia for 20 min followed by 48 h of reperfusion. Since CNPase mediates, at least in part, the renal metabolism of 2′,3′-cAMP to 2′-AMP, the hypothesis was that in CNPase −/− mice, renal outcomes would be worse because of accumulation of 2′,3′-cAMP and diminished production of adenosine. Indeed, analysis of urinary purines indicated attenuated metabolism of 2′,3′-cAMP to 2′-AMP in CNPase −/− mice. Neither genotype nor AKI affected blood pressure, heart rate, urine volume, or albumin excretion. As expected, in CNPase +/+ mice, AKI significantly reduced renal blood flow (as measured by transit-time nanoprobes) and glomerular filtration rate (assessed by ^{14}C-inulin clearance) and increased urinary excretion of kidney injury molecule-1 (a biomarker for proximal tubular damage) and neutrophil gelatinase-associated lipocalin (a general biomarker for severity of kidney damage). Surprisingly, AKI had little effect on these parameters in CNPase −/− mice. A detailed histological analysis revealed that AKI induced severe damage in kidneys from CNPase +/+ mice that included epithelial apoptosis (cell shrinkage, blebbing, nuclear fragmentation, chromatin condensation, phagocytosis), tubular injury (necrosis, degeneration, dilation, interstitial edema), and vasculature injury (peritubular capillary congestion). These renal histological changes induced by AKI were markedly reduced in CNPase −/− mice. Therefore, CNPase (an enzyme involved in the renal 2′,3′-cAMP-adenosine pathway (Jackson et al. 2014b)) rather than protecting against AKI actually augmented renal damage.

Current studies are underway to determine why the 2′,3′-cAMP-adenosine pathway in the kidney is harmful and therefore why blocking renal CNPase is beneficial. Because TNAP is reduced in kidneys undergoing AKI (Peters

et al. 2013) and because renal TNAP is necessary for the metabolism of 2′-AMP to adenosine (Jackson, unpublished data), it is conceivable that in AKI, 2′-AMP accumulates and mediates kidney injury. This would explain in part why preventing the metabolism of 2′,3′-cAMP to 2′-AMP is renoprotective and why administering alkaline phosphatase is renoprotective in AKI (Heemskerk et al. 2009; Pickkers et al. 2012). Indeed, preliminary studies demonstrate that in the isolated, perfused rat kidney, 2′,3′-cAMP and 2′-AMP cause potent and efficacious renal vasoconstriction both in vitro and in vivo but only when kidney TNAP is inhibited so that 2′-AMP is not converted to adenosine and accumulates (Jackson, E.K.: unpublished data). Therefore, inhibition of renal CNPase may protect against AKI in part by preventing the formation of 2′-AMP in TNAP-depleted kidneys, thus improving renal blood flow. Future studies are required to determine how 2′-AMP induces renal vasoconstriction (e.g., stimulation of GPCRs, activation of ion channels, production of reactive oxygen species, etc.). Imaging by transmission electron microscopy of injured CNPase −/− versus injured CNPase +/+ kidneys demonstrated more mitophagy and fewer mitochondria in injured CNPase −/− kidneys (Jackson et al. 2015). Thus, it appears that 2′,3′-cAMP-induced mitochondrial injury in CNPase −/− kidneys allows for more complete removal of damaged mitochondria. Since renal epithelial cells have a large reserve of mitochondria, cell death may be prevented by eliminating leaky, malfunctioning mitochondria despite an overall reduction in the number of mitochondria per cell. Whatever the mechanism, the fact that inhibition of CNPase is renoprotective against AKI is an exciting development. This observation justifies the development of CNPase inhibitors for treatment of AKI, a clinical occurance that is frequent, deadly, and costly and for which there are currently no effective treatments.

5 Other 2′,3′-cNMP-Nucleoside Pathways

It should be emphasized that other 2′,3′-cNMPs-nucleoside pathways may exist and importantly contribute to cell signaling. For example, we recently discovered that 2′,3′-cGMP, 2′-GMP, 3′-GMP, and guanosine are excreted into the urine by mouse kidneys in vivo (Jackson, E.K., unpublished observation). Moreover, in CNPase knockout mice, we observed that the urinary excretion of 2′-GMP is reduced, whereas the urinary excretion of 2′,3′-cGMP and 3′-GMP is increased (Jackson, E.K., unpublished observation). These findings are consistent with the existence of a 2′,3′-cGMP-guanosine pathway in which 2′,3′-cGMP is metabolized to 2′-GMP (by CNPase) and to 3′-GMP. Likely, 2′-GMP and 3′-GMP would be further metabolized to guanosine. 2′,3′-cGMP appears to have pharmacological activity because, like 3′,5′-cGMP, 2′,3′-cGMP stimulates ATPase activity and is transported (probably by MRP5) in human erythrocytes (Boadu et al. 2001). It is conceivable that 2′-GMP or 3′-GMP per se has biological activity. Moreover, recent studies reveal that extracellular guanosine increases the levels of extracellular adenosine by interfering with adenosine disposition mechanisms (Jackson et al. 2013, 2014a; Jackson and Gillespie 2013b; Jackson and Mi 2014). Therefore, one could envision

a convergence of the extracellular 2′,3′-cAMP-adenosine pathway with the extracellular 2′,3′-cGMP-guanosine pathway resulting in enhanced signaling by adenosine due to the guanosine-adenosine interaction. Although yet to be studied, it is also conceivable that 2′,3′-cUMP is converted to 2′-UMP and 3′-UMP and then to uridine. Because uridine has biological activity (Connolly and Duley 1999; Kimura et al. 2001; Dobolyi et al. 2011), this too could be an important 2′,3′-cNMP-nucleoside pathway.

6 Conclusion

This review focuses on the discovery of 2′,3′-cNMPs, with emphasis on 2′,3′-cAMP and its involvement in the 2′,3′-cAMP-adenosine pathway. The current working model is that tissue injury stimulates RNA breakdown and this initiates the 2′,3′-cAMP-adenosine pathway which in the brain may protect against further injury by reducing levels of intracellular 2′,3′-cAMP and increasing levels of extracellular adenosine yet in the kidney may have the opposite effect by generating a renal vasoconstrictor (2′-AMP) and by inhibiting mitophagy of damaged mitochondria. With regard to 2′,3′-cNMPs and their downstream metabolites, there is no doubt much to be discovered.

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8-Nitro-cGMP: A Novel Protein-Reactive cNMP and Its Emerging Roles in Autophagy

Hirokazu Arimoto and Daiki Takahashi

Contents

Abstract

Nitric oxide (NO) raises the intracellular 3′,5′-cyclic guanosine monophosphate (cGMP) level through the activation of soluble guanylate cyclase and, in the presence of reactive oxygen species (ROS), reacts with biomolecules to produce nitrated cGMP derivatives. 8-Nitro-cGMP was the first endogenous cGMP

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derivative discovered in mammalian cells (2007) and was later found in plant cells. Among the six nitrogen atoms in this molecule, the one in the nitro group (NO_2) comes from NO. This chapter asserts that this newly found cGMP is undoubtedly one of the major physiological cNMPs. Multiple studies suggest that its intracellular abundance might exceed that of unmodified cGMP. The characteristic chemical feature of 8-nitro-cGMP is its ability to modify proteinous cysteine residues via a stable sulfide bond. In this posttranslational modification, the nitro group is detached from the guanine base. This modification, termed "protein *S*-guanylation," is known to regulate the physiological functions of several important proteins. Furthermore, 8-nitro-cGMP participates in the regulation of autophagy. For example, in antibacterial autophagy (xenophagy), *S*-guanylation accumulates around invading bacterial cells and functions as a "tag" for subsequent clearance of the organism via ubiquitin modifications. This finding suggests the existence of a system for recognizing the cGMP structure on proteins. Autophagy induction by 8-nitro-cGMP is mechanistically distinct from the well-described starvation-induced autophagy and is independent of the action of mTOR, the master regulator of canonical autophagy.

Keywords
8-Amino-cGMP • 8-Nitro-cGMP • 8-SH-cGMP • Autophagy • *S*-Guanylation • Ubiquitination

1 Discovery of 8-Nitro-cGMP

1.1 Detection of 8-Nitro-cGMP

Endogenous nitric oxide (NO) and reactive oxygen species (ROS) are implicated in the nitration of biomolecules such as amino acids and fatty acids. Guanine nitration at position 8 of the ring has also been investigated since the 1990s as an inflammation marker because inducible nitric oxide synthase (iNOS) is upregulated in inflammation (Yermilov et al. 1995; Spencer et al. 1996; Ohshima et al. 2006).

In the early 2000s, Akaike et al. speculated that 3′,5′-cyclic guanosine monophosphate (cGMP), an important second messenger, may have an endogenous derivative with such a nitro group. Detailed analysis using chemically prepared authentic 8-nitro-cGMP was culminated in the discovery of endogenous 8-nitro-cGMP in cultured cells (Sawa et al. 2007) (Fig. 1).

The intracellular 8-nitro-cGMP concentration can be estimated by immunocytochemistry or mass spectrometry (MS). However, the accurate quantification of 8-nitro-cGMP in a biological sample was not trivial during the early research period because of its instability during sample preparation. Prior addition of isotopically (^{15}N)-labeled 8-nitro-cGMP before extraction was found to minimize the loss of endogenous 8-nitro-cGMP in the sample (Fujii et al. 2010). This isotope-labeled 8-nitro-cGMP not only prevents the degradation of endogenous 8-nitro-cGMP

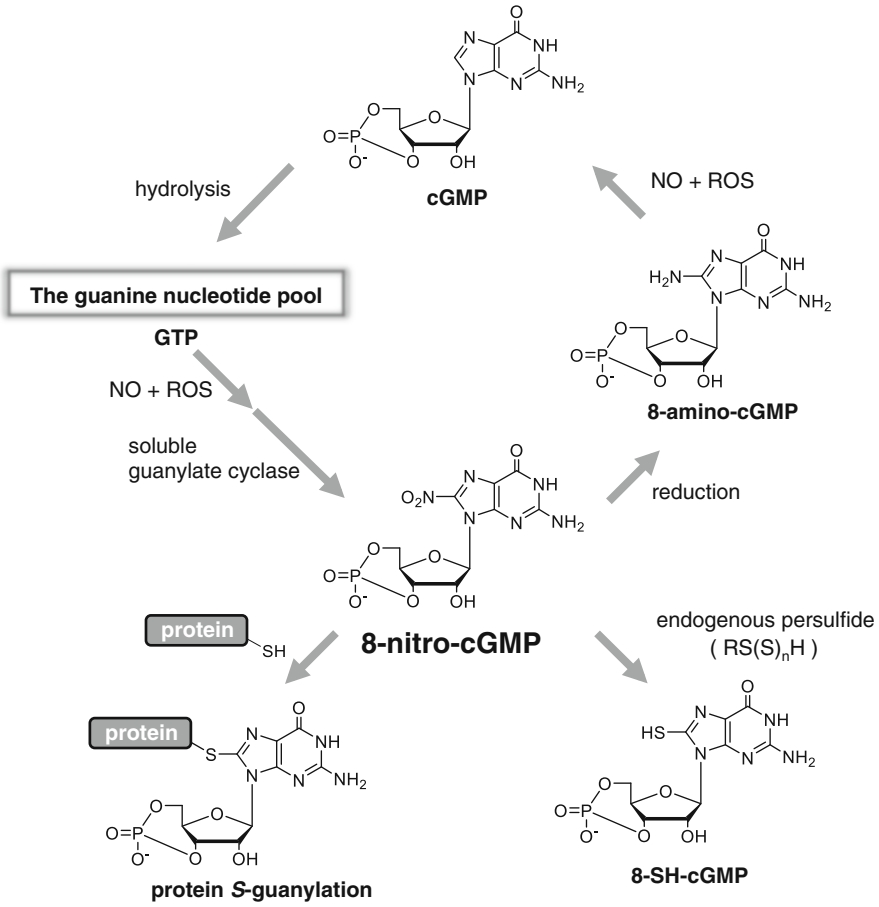

Fig. 1 Formation and metabolisms of 8-nitro-cGMP. Endogenous 8-nitro-cGMP forms from the guanine nucleotide pool by the action of nitric oxide (NO) and reactive oxygen species (ROS). This nucleotide either modifies cysteine-containing proteins via "*S*-guanylation" or transforms into 8-amino-cGMP, which is further converted into intact cGMP by NO and ROS. 8-Nitro-cGMP reacts with endogenous persulfides to produce 8-SH-cGMP, whereas the similar reaction with sulfhydryl anion (SH^-) produces 8-amino-cGMP

present in smaller amounts but also serves as an internal standard for mass spectrometric analysis. Notably, the endogenous level of 8-nitro-cGMP was initially estimated to be less than 1% in all cGMPs (Sawa et al. 2007), but recent results using this isotope labeling technique showed that the intracellular 8-nitro-cGMP concentration is often higher than unmodified cGMP (Fujii et al. 2010). Moreover, 8-nitro-cGMP may be widely distributed in animal tissues because it was recently found in both mouse heart (Nishida et al. 2012) and brain (Kunieda et al. 2015).

It is now generally accepted that 8-nitro-cGMP is biosynthesized via the nitration of GTP and subsequent action of guanylate cyclase (Fujii et al. 2010; Ahmed

et al. 2012). In studies using rat C6 cells activated by lipopolysaccharide (LPS), the direct formation of 8-nitro-cGMP from cGMP appeared unlikely, because the endogenous cGMP concentration (maximum 4 μM) was consistently lower than 8-nitro-cGMP (maximum 40 μM). Furthermore, peroxynitrite, a nitration agent, quickly reacts with guanosine phosphates in the order GTP>GDP>GMP>cGMP. In addition, 8-nitro-GTP is a good substrate of the soluble guanylate cyclase in vitro yielding 8-nitro-cGMP, and an inhibitor of soluble guanylate cyclase was shown to suppress intracellular 8-nitro-cGMP generation.

1.2 *S*-Guanylation: A Novel Posttranslational Modification (PTM) by 8-Nitro-cGMP

The analysis of endogenous 8-nitro-cGMP was difficult in earlier studies because of its instability in biological samples. Because the cyclic phosphate of 8-nitro-cGMP is resistant to hydrolysis by PDE1 and PDE5 (Sawa et al. 2007), other degradation pathways may account for its instability. An important clue to the identity of endogenous 8-nitro-cGMP degradation pathways was obtained in 2005. Akaike and our group noticed that the yellow color of 8-nitro-cGMP disappeared upon treatment with thiol-containing compounds. A model reaction with 8-nitro-cGMP and glutathione was performed in vitro at room temperature, and the reaction product was thoroughly investigated using two-dimensional nuclear magnetic resonance (NMR) techniques and MS. These analyses revealed a glutathione adduct at the 8 position of the guanine ring. This substitution of the nitro group with thiol had never been reported in the chemistry literature and so was quite astonishing. This reaction was named "protein *S*-guanylation" (Fig. 1).

Polyclonal and monoclonal antibodies specific to protein *S*-guanylation were developed for immunoblot analysis and immunohistochemistry. The antibodies demonstrated that this PTM is present in cells under basal conditions and that levels increase during inflammation. Regulatory mechanisms of this PTM are currently unclear because *S*-guanylation proceeds without enzymes. The reactivity of each cysteine residue varies considerably depending on the neighboring sequence. For example, Kelch-like ECH-associated protein (Keap1), which contains multiple reactive cysteine residues, was *S*-guanylated even in the presence of a 1,000-fold excess of glutathione (Sawa et al. 2007). Thus, the propensity for *S*-guanylation may depend, at least in part, on the chemical environment around cysteine residues.

As described in Sects. 3 and 4, protein *S*-guanylation is involved in several physiological processes. However, it is still uncertain if endogenous levels and distributions of NO and ROS alone can account for the spatiotemporal dynamics of this PTM. Similarly, the intrinsic reactivity of each cysteine residue may not be the only factor governing the selectivity of *S*-guanylation. An interesting topic for future studies is the possible participation of enzymes.

2 Metabolism: Reduction to Intact cGMP via 8-Amino-cGMP/8-SH-cGMP

To date, three metabolic pathways for 8-nitro-cGMP have been elucidated, including protein *S*-guanylation. Here we describe two additional pathways (Fig. 1).

2.1 Reductive Metabolic Pathway to Unmodified cGMP via 8-Amino-cGMP

^{18}O-labeled 8-nitro-cGMP was chemically synthesized, and its metabolic fates in cultured cells were monitored by liquid chromatography–mass spectrometry (LC-MS). Along with the *S*-guanylation products containing glutathione or cysteine, a new nucleotide, 8-amino-cGMP (8-NH_2-cGMP), was identified (Saito et al. 2012). Immunocytochemistry of LPS-activated mouse macrophage-like RAW264.7 cells using a specific 8-amino-cGMP antibody revealed that the endogenous degradation of 8-nitro-cGMP was accompanied by the formation of 8-amino-cGMP. Moreover, isotope-labeled 8-amino-cGMP was further converted to unmodified cGMP in LPS-treated cells. It is intriguing that both oxidative modification (guanine nitration) and reductive metabolism (cGMP formation) occur simultaneously under oxidative stress conditions.

2.2 Substitution of the Nitro Group by Persulfide Species: 8-SH-cGMP Formation

Based on the analogy to *S*-guanylation at thiols in cysteine residues, substitution with endogenous sulfur species may be expected. Hydrogen sulfide (H_2S) is one such sulfur species attracting considerable attention as a gaseous signaling mediator. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are responsible for H_2S biosynthesis. Knockdown of these enzymes resulted in increased 8-nitro-cGMP levels, indicating the regulation of 8-nitro-cGMP actions by sulfur species. Moreover, a plausible product of *S*-guanylation with H_2S, 8-mercapto-cGMP (8-SH-cGMP), was identified in mammalian cell lysates by LC-MS analysis (Nishida et al. 2012).

However, the involvement of H_2S as a precursor of 8-SH-cGMP was soon questioned because the reaction of 8-nitro-cGMP with the hydrogen sulfide anion (SH^-) in vitro predominantly yields 8-amino-cGMP (8-NH_2-cGMP) (Terzić et al. 2014), a previously identified metabolite of 8-nitro-cGMP (Saito et al. 2012). Thus, it is reasonable to conclude that endogenous H_2S may serve as a reducing agent in the conversion of 8-nitro-cGMP to 8-amino-cGMP.

A recent paper from the Akaike group provided an alternative pathway for 8-SH-cGMP biosynthesis. They demonstrated that CBS and CSE produce persulfide species (e.g., RS-S-H) that are more nucleophilic than hydrogen sulfide. Specifically, persulfides of cysteine (Cys-S-S-H) and glutathione (GS-S-H) are

produced that react with 8-nitro-cGMP to provide the substitution product (RS-S-cGMP), which is expected to yield 8-SH-cGMP via a thiol-disulfide exchange reaction (Ida et al. 2014; Terzić et al. 2014).

Treatment of 8-SH-cGMP in vitro with hydrogen peroxide (H_2O_2) or reactive nitrogen species provided intact cGMP (Nishida et al. 2012). Similarly, 8-amino-cGMP, another metabolite of 8-nitro-cGMP, has already been demonstrated to yield cGMP in LPS-stimulated cells. Thus, 8-SH-cGMP may also be converted to intact cGMP in cells.

3 Regulation of Protein Functions by *S*-Guanylation

3.1 Activation Nrf2-ARE Pathway via *S*-Guanylation of Keap1

Keap1 is a major sensor of oxidative stress. It contains 25 cysteine residues and was the first identified target of protein *S*-guanylation (Sawa et al. 2007). Keap1 facilitates Nrf2 proteasomal degradation by acting as an adaptor for Cullin3-based E3 ubiquitin ligase (Kobayashi et al. 2004). Conversely, *S*-guanylation of Keap1 cysteine residues at the Nrf2 interaction site causes Nrf2 to dissociate from Keap1 and translocate into the nucleus, leading to the transcriptional activation of cytoprotective genes (Fig. 2). The cytoprotective actions of 8-nitro-cGMP against oxidative stress have been directly demonstrated (Cosker et al. 2014).

It should be emphasized, however, that many other endogenous electrophiles also modify the cysteine residues of Keap1 and exhibit similar cytoprotective actions. Moreover, 8-nitro-cGMP is an electrophile with modest reactivity; therefore, its contribution to Keap1 modification and downstream anti-oxidative stress responses requires further study.

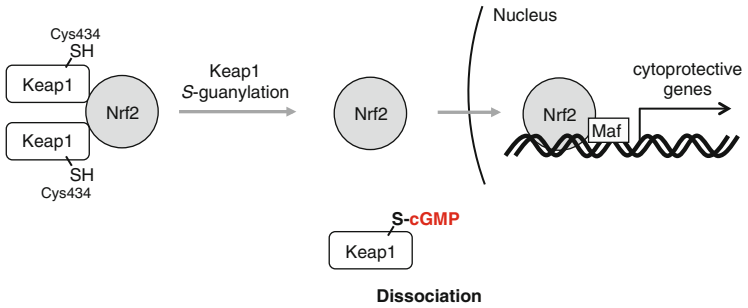

Fig. 2 Keap1 *S*-guanylation activates Nrf2-ARE pathway. The Keap1-Nrf2 pathway regulates more than hundreds of cytoprotective genes. Keap1 inactivation by *S*-guanylation at cysteine residues leads to Nrf2 nuclear translocation and induction of the cytoprotective machineries. *Keap1* Kelch-like ECH-associated protein 1, *Nrf2* nuclear erythroid 2-related factor 2, *ARE* antioxidant response element

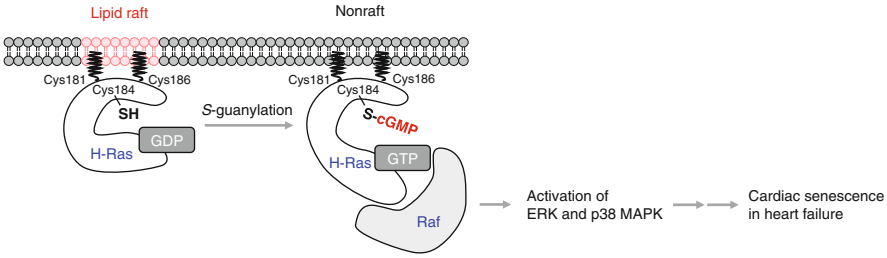

Fig. 3 *S*-Guanylation of oncogenic H-Ras activates Raf-dependent ERK and p38 MAPK pathways. Partitioning of H-Ras between raft and non-raft domains is affected by the *S*-guanylation at Cys184. Non-raft-resident GTP-bound H-Ras activates Raf1 and the downstream signaling pathways. Cys181 and Cys186 of H-Ras are modified by palmitoylation and isoprenylation, respectively

3.2 Cellular Senescence by *S*-Guanylation of H-Ras Protein in a Heart Failure Model

In the early stages of heart failure, iNOS is overexpresssed to trigger cytoprotective responses. On the other hand, iNOS also serves as a crucial factor for cardiac remodeling. To understand these apparently opposing effects, the actions of 8-nitro-cGMP on rat primary cardiomyocyte and cardiac fibroblast cultures were investigated (Nishida et al. 2012). Exogenous 8-nitro-cGMP treatment induced growth arrest and senescence, as measured by endogenous β-galactosidase activity. These effects were not observed with other cGMP derivatives. The accumulation of 8-nitro-cGMP in mouse heart after myocardial infarction (MI) also suggests a role in the pathogenesis of heart failure. The administration of low-dose sodium hydrosulfide (50 μmol/kg/day) reduced the accumulation of 8-nitro-cGMP and limited the dysfunction of the left ventricle after MI. Oncogenic H-Ras activation can also induce cellular senescence, and Cys184 *S*-guanylation is a crucial regulator because 8-nitro-cGMP did not induce senescence in a rat cardiac fibroblast mutant expressing Cys ^{C184S} H-Ras (Fig. 3).

3.3 *S*-Guanylation in the Mitochondria and Its Effects on ROS Export

The identification of new *S*-guanylated proteins provides additional clues to the underlying functions of 8-nitro-cGMP. Two optimized procedures for "*S*-guanylation proteomics" were reported (Rahaman et al. 2014), each with specific merits and demerits. In the first procedure, the immunoaffinity capture and LC-MS/MS method, protein samples are digested directly, and the resulting *S*-guanylated peptides are collected by immunoaffinity capture, followed by identification using

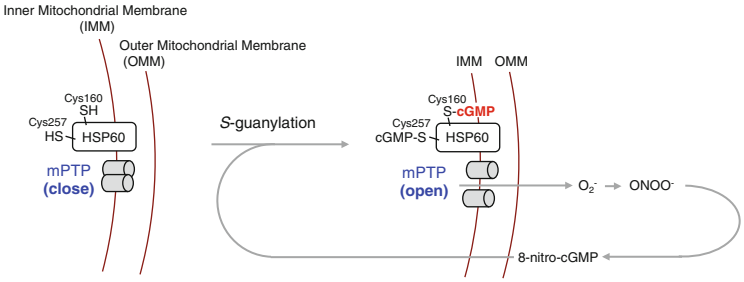

Fig. 4 Protein *S*-guanylation in the mitochondria regulates ROS export. *S*-guanylation of mitochondrial heat-shock protein 60 (HSP60) at Cys160 and Cys257 was suggested to regulate opening of mitochondrial permeable-transition pore (mPTP) and release of ROS into the cytoplasm. O_2^-: superoxide anion radical

LC–tandem MS. This procedure generally requires relatively large protein samples (1 mg/analysis). In the other procedure, the protein samples are first separated by 2D-PAGE, and the *S*-guanylated spots are excised and digested for LC-MS/MS analysis. In this 2D-PAGE-based procedure, smaller samples can be used (0.1 mg protein/analysis), and the relative abundance of each *S*-guanylated protein spots is known before MS/MS analysis. However, 2D-PAGE is insensitive for lipophilic membrane proteins and basic proteins (pI > 8).

Using these procedures, several mitochondrial proteins, including Hsp60 and mortalin, were shown to be *S*-guanylated in cells after stimulation with LPS and cytokines. Subsequent studies indicated that these PTMs may be involved in mitochondrial permeability-transition pore (mPTP) opening and mPTP-mediated ROS release into the cytoplasm (Fig. 4).

3.4 8-Nitro-cGMP in Nerve Cells

Neuronal NO synthase (nNOS) is also involved in 8-nitro-cGMP formation (Kasamatsu et al. 2014). Calcium ionophore treatment of nNOS-transfected HEK-293 cells enhanced protein *S*-guanylation suggesting 8-nitro-cGMP formation by nNOS-derived NO and ROS in the nervous system. In a nerve terminal model (synaptosomes), *S*-guanylation of synaptosomal-associated protein 25 (SNAP25) was identified (Kunieda et al. 2015). SNAP25 is a member of a SNARE complex that regulates synaptic vesicle fusion for exocytosis. Cysteine 90 of SNAP25 was identified as the main site of *S*-guanylation, and this modification was shown to stabilize the SNARE complex (Fig. 5).

Another study using primary mesencephalic cells showed that exogenous 8-nitro-cGMP protected dopaminergic neurons from cytotoxic 1-methyl-4-phenylpyridinium (MPP^+) via heme oxygenase-1 formation as well as the canonical protein kinase G (PKG) signaling pathway. Elevated extracellular K^+ concentration

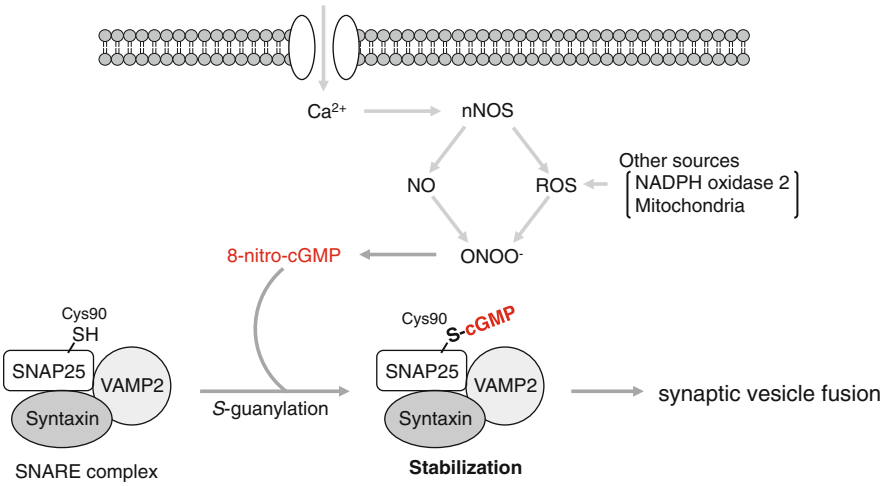

Fig. 5 Protein *S*-guanylation stabilizes SNARE complex. Synaptosomal-associated protein 25 kDa (SNAP25) is a component of the SNARE complex, which mediates fusion of vesicles with membranes. Ca^{2+}-dependent activation of neuronal NO synthase (nNOS) produces 8-nitro-cGMP, and the resulting *S*-guanylation of SNAP25 at Cys90 enhances the stability of SNARE complex. This stabilizing effect of the SNARE complex may promote synaptic vesicle fusion

induced an endogenous formation of 8-nitro-cGMP and also resulted in neuroprotection (Kurauchi et al. 2013). These studies suggest that nNOS-derived 8-nitro-cGMP has an important cytoprotective function in neuronal cells.

3.5 8-Nitro-cGMP in Plants

As described elsewhere in this book, the roles of cGMP in plants are not well understood compared to those in animals. Recently, the existence of 8-nitro-cGMP in stomatal guard cells of *Arabidopsis* was described (Joudoi et al. 2013) (Fig. 6). 8-Nitro-cGMP and cGMP appear to have different effects on stomatal function. Although 8-nitro-cGMP induced stomatal closure, 8-Br-cGMP, a widely used membrane permeable analogue of cGMP, did not. In the dark, 8-Br-cGMP induced stomatal opening, but 8-nitro-cGMP did not. Judoi et al. showed that the effects of 8-nitro-cGMP signaling are mediated by Ca^{2+}, cyclic adenosine-5´-diphosphate-ribose, and the SLOW ANION CHANNEL1 (SLAC1). The same research group recently reported that the 8-nitro-cGMP metabolite 8-SH-cGMP (8-mercapto-cGMP, Sect. 2.2) also induces stomatal closure (Honda et al. 2015). However, the direct involvement of protein *S*-guanylation in these effects has not been demonstrated.

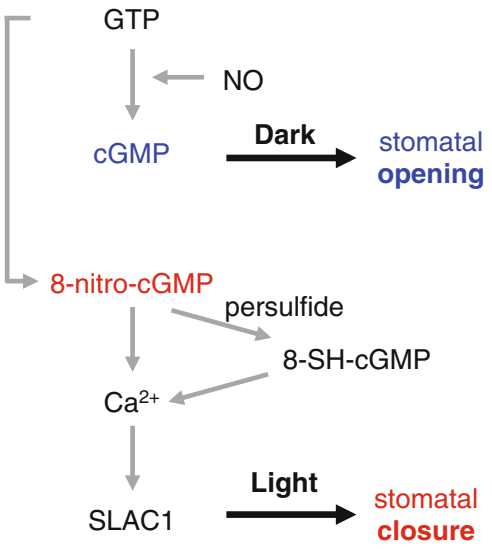

Fig. 6 8-Nitro-cGMP in plants. 8-nitro-cGMP and cGMP have different effects on regulation of stomatal function. 8-Nitro-cGMP causes stomatal closure, whereas 8-Br-cGMP, a widely used membrane permeable analogue of cGMP, does not. In the dark, 8-nitro-cGMP does not cause stomatal opening. *SLAC* slow anion channel

4 8-Nitro-cGMP in Autophagy Regulation

Voluminous evidence accrued over the past decade clearly indicates that 8-nitro-cGMP can covalently modify Cys-containing proteins and influence their function. Recently, autophagy induction was demonstrated to be another major function of 8-nitro-cGMP (Ito et al. 2013; Rawet-Slobodkin and Elazar 2013; Abada and Elazar 2014).

4.1 8-Nitro-cGMP as an Endogenous Autophagy Inducer

Autophagy is one of the two major cellular degradation systems conserved across eukaryotes (Fig. 7). A double-membrane vesicle called the autophagosome engulfs autophagic cargos and subsequently fuses with the lysosome (Mizushima and Komatsu 2011) for eventual degradation and recycling. In mammals, autophagy can be monitored by labeling the autophagosomal LC3 protein (homolog of yeast Atg8). Autophagy is a well-described response to nutrient starvation but is also induced by other stimuli. Damaged organelles, protein aggregates, and pathogens can be selectively destroyed by separate autophagic pathways that differ mechanistically from the canonical starvation-induced autophagic pathway.

LPS is a component of bacterial cell membranes and a stimulator of innate immunity, eliciting a variety of inflammatory responses. LPS also induces autophagy, and, in this specific case, autophagy was shown to depend on NO and ROS (Yuan et al. 2009). Because the intracellular 8-nitro-cGMP level is upregulated by LPS-induced inflammation (Sawa et al. 2007), the involvement of

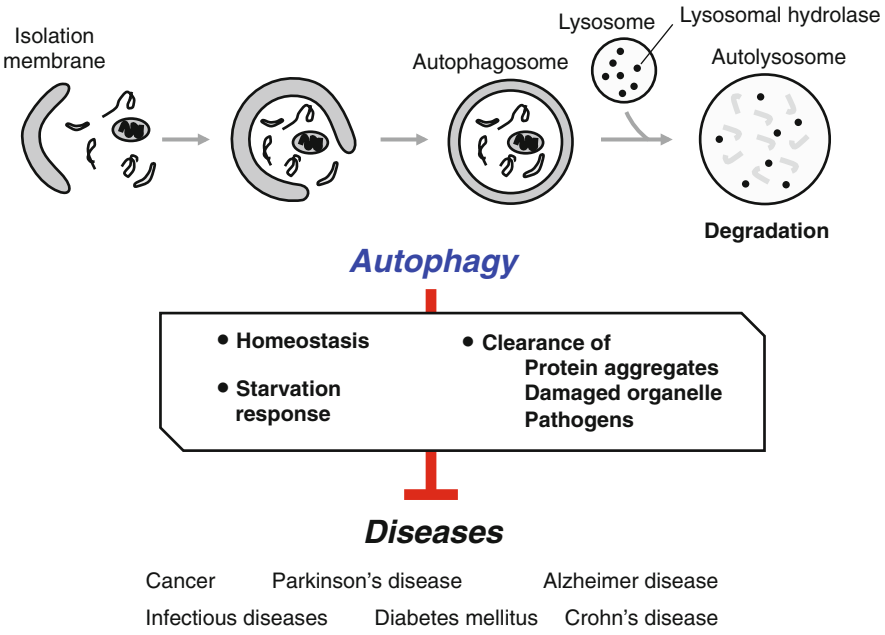

Fig. 7 Autophagy suppresses diseases via intracellular degradation. Autophagy is a cellular degradation system that plays a major role in human health and disease. Autophagosome surrounds and sequesters cytoplasmic cargos for lysosomal degradation. Autophagy is associated with suppression of diseases including cancer and neurodegenerative diseases

this cGMP analogue in autophagy regulation was examined (Ito et al. 2013). Indeed, exogenous 8-nitro-cGMP treatment (50–100 μM) increased LC3-positive puncta in cells suggesting autophagy induction, and no cytotoxicity was observed. Considering the fact that cGMP does not penetrate cell membranes, the observed membrane permeability of 8-nitro-cGMP is noteworthy for future research and possibly for clinical applications.

An initial mechanistic study by Ito et al. demonstrated that 8-nitro-cGMP-induced autophagy does not employ target of rapamycin (TOR), the master regulator of canonical starvation-induced autophagy. Immunocytochemical analysis showed that *S*-guanylated proteins partly co-localized with LC3-positive autophagosomes, suggesting that 8-nitro-cGMP not only induces autophagy but also participates in selective clearance of its targets (*S*-guanylated proteins).

4.2 *S*-Guanylation as a Tag for Degradation in Antibacterial Autophagy

In contrast to the nonselective nature of canonical starvation-induced autophagy, autophagy for the clearance of intracellular pathogens or damaged organelles is

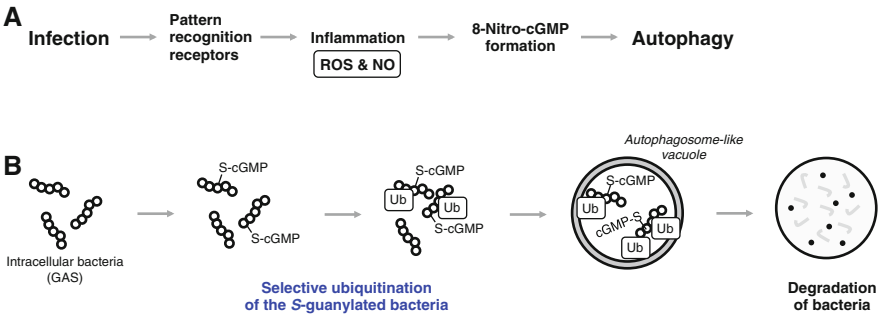

Fig. 8 Endogenous 8-nitro-cGMP accelerates selective autophagic clearance of invading group A streptococcus (GAS). (**a**) The innate immune system functions to trigger inflammation upon sensing of conserved microbial structures of invading pathogens. Enhanced formation of 8-nitro-cGMP during inflammation activates autophagy. (**b**) Ito et al. (2013) reported that accumulation of *S*-guanylated proteins around bacteria is the tag for subsequent ubiquitination and clearance. Involvement of the ubiquitin modification has been attracting attention as the degradation tag, but the correlation of *S*-guanylation and ubiquitination was demonstrated for the first time. *Ub* polyubiquitin chain

considered highly selective. Because bacterial infection elevates the intracellular formation of 8-nitro-cGMP, the contribution of 8-nitro-cGMP to antibacterial autophagy was investigated using group A streptococcus (GAS) and murine macrophages. Autophagic clearance of GAS had previously been described in HeLa cells (Nakagawa et al. 2004). Invading GAS was efficiently cleared also from murine macrophages via autophagy, and this process required endogenous 8-nitro-cGMP generation (Ito et al. 2013) (Fig. 8). Exogenous addition of 8-nitro-cGMP to the culture media further accelerated GAS clearance.

Selective autophagy is currently a field of extensive research, and autophagic cargo ubiquitination is also attracting attention because many "autophagy adaptors" have the ability to connect ubiquitinated cargos with LC3 protein on the autophagosomal membrane. An important question here is how specific autophagic cargos are recognized and selectively modified by ubiquitin ligases. We observed the accumulation of *S*-guanylation in GAS-containing autophagosomes (Fig. 8b). This *S*-guanylation of GAS cells was the tag required for subsequent modification with K63-linked polyubiquitin chains. Thus, *S*-guanylation is the degradation tag for antibacterial autophagy of GAS (Ito et al. 2013). Inhibitors of endogenous 8-nitro-cGMP generation could thus suppress the autophagic clearance of the GAS bacteria. Some of these *S*-guanylated bacterial proteins were identified by LC-MS analysis; however, host-derived *S*-guanylated proteins may also be involved in GAS recognition in autophagosomes.

As mentioned in previous sections, 8-nitro-cGMP can form in cells even in the absence of bacterial infection. K63-linked polyubiquitination was also involved in autophagy induction under these conditions (Ito et al. 2013), suggesting that

S-guanylation is a general tag for autophagic degradation. However, many unanswered questions still exist. For example, can a protein be both *S*-guanylated and ubiquitinated? Or can a putative "*S*-guanylation recognition protein" bind to a *S*-guanylated protein and be ubiquitinated? Also, what are the major physiological substrates of this selective degradation?

Autophagy is believed to suppress a variety of human diseases; therefore, considerable efforts have been made to identify or create small molecular weight autophagy regulator (Levine et al. 2015; Rubinsztein et al. 2015). Among known autophagy inducers, 8-nitro-cGMP is the only compound that induces a selective degradation of its targets. Moreover, 8-nitro-cGMP is membrane permeable and resists hydrolysis by phosphodiesterase (Sawa et al. 2007). This nucleotide is thus a promising lead drug for a clinical activator of autophagy.

5 Preparative Methods of 8-Nitro-cGMP

As of late 2015, 8-nitro-cGMP is not available from commercial sources; therefore, researchers must prepare this nucleotide for their experiments. 8-Nitro-cGMP is believed to be generated in vivo via GTP nitration by reactive nitrogen species (e.g., peroxynitrate, Sect. 1.1). For preparative purposes in vitro, however, this nitration reaction is not practical because of its low yield. Instead, commercially available 8-bromo-cGMP (8-Br-cGMP) is used as the starting material, and nitration is performed with sodium nitrite ($NaNO_2$) (Sawa et al. 2007). In our hands, this protocol yields an average of 10–20% 8-nitro-cGMP from a 100-mg scale reaction (Fig. 9). Unfortunately, this method is not easily scalable for more material because it requires HPLC purification of the product from unreacted 8-Br-cGMP. Thus, development of alternative, scalable synthetic procedures is in great demand for the pharmaceutical application of 8-nitro-cGMP in animal models. Organic chemists are also working on developing chemical tools such as fluorescent probes to study the functions of 8-nitro-cGMP (Saito et al. 2013; Samanta et al. 2014).

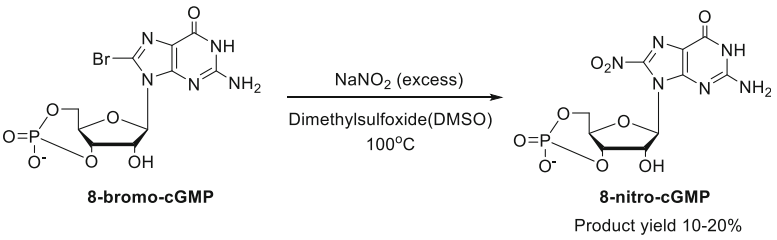

Fig. 9 8-Nitro-cGMP preparation from commercially available 8-bromo-cGMP

6 Future Problems

6.1 Regulatory Mechanism of Protein *S*-Guanylation

The majority of 8-nitro-cGMP effects described to date appear to be mediated by protein *S*-guanylation. However, no regulatory mechanism has been reported that governs the spatiotemporal selectivity of the modification. A current explanation for the selectivity is the cysteine acidity (pK_a) of each site within each target protein. Neighboring amino acid residues are known to affect the acidity. Cysteine residues with low pK_a dissociate to form sulfur anions that are more reactive with 8-nitro-cGMP, at least in vitro. The steric environment of each cysteine residue may also affect its reactivity.

However, it is unclear whether cysteine acidity alone can confer the requisite target specificity for intracellular signaling and autophagy regulation; therefore, the existence of enzymes that catalyze protein *S*-guanylation is a crucial future issue in this field.

6.2 Physiological Importance of 8-nitro-cGMP in PKG-Mediated Signaling Pathways

This chapter has focused on properties unique to the nitrated cGMP. However, it should be emphasized that like cGMP, 8-nitro-cGMP retains the capacity to activate PKG (Sawa et al. 2007). According to several studies, endogenous 8-nitro-cGMP levels are similar or even higher under some conditions (e.g., infection/inflammation) than cGMP levels (Fujii et al. 2010; Kunieda et al. 2015). The contribution of 8-nitro-cGMP to PKG-mediated signaling is worthy of further investigation. The metabolites of 8-nitro-cGMP (8-amino-cGMP and 8-SH-cGMP) also need to be considered as physiological PKG activators.

7 Concluding Remarks

8-Nitro-cGMP is the most recently discovered endogenous cNMP, and there is compelling evidence that endogenously produced 8-nitro-cGMP acts as a physiological modulator through the PTM of proteins and possibly also by PKG activation. It exists not only in mammals but also in higher plants, and the endogenous concentration often exceeds that of cGMP. This chapter is by no means a comprehensive discussion of all known or postulated physiological functions because a very large number of proteins can be modified by this cNMP. The possible pharmaceutical application of this nucleotide for a variety of autophagy-related human disorders, such as neurodegenerative diseases and cancer, is thus a promising avenue for future research.

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Cyclic Dinucleotides in the Scope of the Mammalian Immune System

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Abstract

First discovered in prokaryotes and more recently in eukaryotes, cyclic dinucleotides (CDNs) constitute a unique branch of second messenger signaling systems. Within prokaryotes CDNs regulate a wide array of different biological processes, whereas in the vertebrate system CDN signaling is largely dedicated to activation of the innate immune system. In this book chapter we summarize the occurrence and signaling pathways of these small-molecule second messengers, most importantly in the scope of the mammalian immune system. In this regard, our main focus is the role of the cGAS-STING axis in the context of microbial infection and sterile inflammation and its implications for therapeutic applications.

Keywords

cGAMP • cGAS • Cyclic dinucleotides • Innate immunity • STING

1 Introduction

Small nucleotide molecules constitute a common branch of second messenger signaling systems present in bacterial, archaeal, and eukaryotic cells (Pesavento and Hengge 2009), classical examples being the well-studied nucleotide second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in eukaryotes (Pesavento and Hengge 2009). Functionally, these molecules have an impact on a plethora of biological processes such as cellular proliferation, differentiation, and cell death, also within cells of the immune system. Another, yet more specialized class of nucleotide second messenger system is based on 2′-5′-oligoadenylates that are synthesized by the family of nucleotidyl transferase 2′-5′-oligoadenylate synthetases (OAS). OAS enzymes are activated by viral double-stranded RNA to produce 2′-5′-oligoadenylates, which in turn activate the latent ribonuclease RNase L. Active RNase L degrades viral and host RNA species to inhibit viral propagation. Finally, an additional nucleotide messenger system is based on cyclic dinucleotides (CDNs), which were initially discovered in prokaryotes. With the identification of CDNs serving as potent activators of the innate immune system and the discovery of a unique metazoan CDN as a dedicated second messenger molecule in innate immune signaling, CDNs have taken center stage in immunology. This chapter focuses on the activation of the mammalian immune system by CDNs and its possible implications in disease and therapeutic applications.

2 Cyclic Dinucleotides: From Prokaryotes to Eukaryotes

2.1 Cyclic-di-GMP

The first reports cataloguing the existence of cyclic dinucleotides arose from studies in the gram-negative bacterium *Gluconacetobacter xylinus* (Ross et al. 1987), where it was found to regulate the biosynthesis of cellulose. Next to its specific function in regulating cellulose synthesis in this prokaryote, subsequent studies revealed that c-di-GMP molecules constitute a ubiquitous second messenger system that is present in many bacterial phyla, where they regulate a wide array of different biological processes (Fig. 1). In fact, c-di-GMP can be described as a life style sensor that controls motility/sessility, biofilm formation, cell cycle, and also virulence in response to external stimuli. Following its initial description in 1987, it took more than a decade to elucidate how c-di-GMP is synthesized and how it is degraded (for detailed reviews see (Pesavento and Hengge 2009; Romling et al. 2013)). As it turned out, the synthesis of c-di-GMP depends on the activity of a diguanylate cyclase that condenses two molecules of GTP in a two-step reaction via an intermediate linear triphosphate pppGpG molecule. The catalytically active amino acids are encoded in the GGDEF protein domain, wherein two GGDEF domains, with each one binding one GTP molecule, cooperatively synthesize

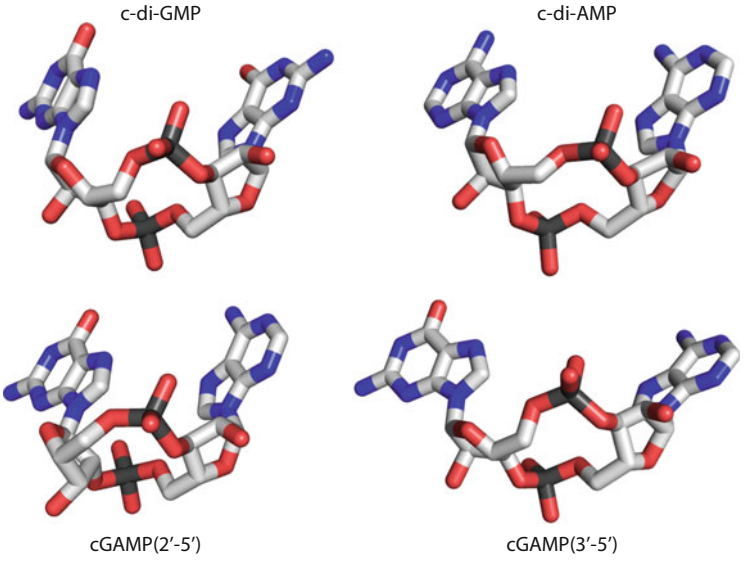

Fig. 1 Structure models of cyclic dinucleotides. Overview of cyclic dinucleotides as they occur in different crystal structures shown as stick representation (c-di-GMP from pdb 2RDE (Benach et al. 2007), c-di-AMP from pdb 4YXM (Muller et al. 2015), cGAMP(2′-5′) from pdb 4KSY (Zhang et al. 2013), 3′,3′-cGAMP pdb 5CFM (Kranzusch et al. 2015))

c-di-GMP. Degradation of c-di-GMP, on the other hand, is carried out by two structurally and functionally distinct types of phosphodiesterases that encode either EAL domains or HD-GYP domains. EAL domain-containing proteins comprise 3′-5′-phosphodiesterase activity, hydrolyzing c-di-GMP into the linear product pGpG. HD-GYP domains, on the other hand, hydrolyze c-di-GMP to two GMP molecules in two subsequent steps (Table 1).

Nearly two decades since the original identification of c-di-GMP, the PilZ domain in the *E. coli* protein YcgR was identified as one of the most important ligand-binding domains for c-di-GMP (Ryjenkov et al. 2006). Since then a number of additional domains have been identified as sensing domains for c-di-GMP (for an overview see (Schaap 2013)). In many cases, c-di-GMP binding induces an allosteric switch in the respective target proteins, thereby modulating enzymatic activity. Additionally, c-di-GMP has also been shown to modulate gene expression at the transcriptional level by directly binding to transcription factors. Moreover, riboswitches within 5′ UTRs of bacterial mRNA can bind c-di-GMP and thereby control transcription and translation of respective mRNAs.

More recently it was found that c-di-GMP production is not only confined to the bacterial world, but also found in the eukaryote *Dictyostelium*, a social amoeba (Chen and Schaap 2012). Identification of the conserved GGDEF domain, encoded by the DgcA gene, within the *D. discoideum* genome was the first hint that these species might also possess a protein similar to the prokaryote diguanylate cyclases. In the absence of adequate nutrition, individual Dictyostelia aggregate to form multicellular motile slugs that subsequently give rise to so-called fruiting bodies. In the context of this process, c-di-GMP is produced, acting as a morphogen that triggers stalk cell differentiation in the fruiting body. The respective sensing domains and possible degradation pathways in this system are currently not known; yet the fact that c-di-GMP does not act as a cell-autonomous second messenger molecule in this context alludes to the possibility that receptor proteins other than the ones known from the prokaryotic world are employed.

Table 1 Cyclic dinucleotides: synthesis and degradation

Cyclic dinucleotide	Synthesis	Degradation	Primary description or review article
c-di-AMP	DAC-domain proteins CdaS, CdaA, DisA	PgpH type (membrane protein) with HD domain, GdpP type (membrane protein)/DhhP type (soluble) with DHH/DHHA1 domains	Commichau et al. (2015), Corrigan and Grundling (2013), and Huynh and Woodward (2016)
c-di-GMP	GGDEF-domain proteins	EAL-domain proteins HD-GYP-domain proteins	Romling et al. (2013) and Hengge (2009)
cGAMP (2′-3′)	DncV	V-cGAP with HD-GYP domain	Davies et al. (2012) and Gao et al. (2015)
cGAMP (2′-5′)	cGAS	ENPP1	Cai et al. (2014) and Li et al. (2014)

2.2 Cyclic-di-AMP

The second class of cyclic dinucleotide molecules being discovered was c-di-AMP (Fig. 1). Surprisingly, c-di-AMP was found as a novel molecule in the course of crystallization studies of the *T. maritima* DisA protein that acts as a checkpoint protein for DNA integrity prior to sporulation (Bejerano-Sagie et al. 2006). DisA builds an octameric protein complex that recognizes branched DNA structures, e.g., Holliday junctions or stalled replication forks. Next to its DNA-binding function, DisA harbors a diadenylyl cyclase (DAC) domain that synthesizes c-di-AMP. Upon recognition of branched DNA substrates, DisA stops synthesizing c-di-AMP, a process that is believed to stall sporulation (Witte et al. 2008). After its discovery, additional studies have revealed that DAC-containing proteins are present in many bacterial phyla, and even archaea. Moreover, direct measurement of c-di-AMP levels proved that considerable amounts of this second messenger exist in a number of bacteria. Next to its role in maintaining DNA integrity in *T. maritima*, *B. subtilis*, and related bacteria, a genome-wide screen (Corrigan et al. 2013) identified a number of additional biological processes that might be controlled by c-di-AMP, most prominently potassium homeostasis and cell wall biogenesis. Indeed, the crucial role for c-di-AMP-dependent signaling is underscored by the fact that the deletion of diadenylyl cyclases has proven unsuccessful in a number of bacteria, rendering c-di-AMP the first essential bacterial second messenger known so far (Commichau et al. 2015). As for c-di-GMP, a series of domains have been identified that confer direct c-di-AMP binding and thereby allosteric regulation of respective domain-containing proteins; however, no common c-di-AMP-binding motif has been identified yet. Moreover, also for c-di-AMP a riboswitch-mediated regulation of transcription and translation has been reported (Nelson et al. 2013). C-di-AMP levels need to be tightly regulated as both too high and too low levels have severe effects on the cells, thus rendering c-di-AMP pathways interesting targets for antimicrobial therapy. This assumes significance as many pathogens like *Listeria*, *MRSA*, and *Mycobacteria* produce c-di-AMP. In analogy to the c-di-GMP system, c-di-AMP signaling is also terminated by two types of phosphodiesterases that degrade c-di-AMP: a membrane-associated HD domain (Huynh et al. 2015) and DHH/DHHA1 domain-containing proteins (Rao et al. 2010) that occur as membrane proteins but also in soluble form (Table 1).

2.3 Cyclic GMP-AMP

Cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) is a hybrid cyclic dinucleotide that was first discovered in *Vibrio cholerae* by Davies and colleagues (Davies et al. 2012) (Fig. 1). Studying the pathogenicity of the 7th pandemic biotype of *V. cholerae*, Davies et al. identified a novel class of dinucleotide cyclases (DncV) as a virulence factor on pandemic island-1. DncV was required for efficient *V. cholerae* intestinal colonization but also regulated the colonization-influencing process of chemotaxis. While DncV was able to

synthesize c-di-GMP or c-di-AMP in the presence of GTP or ATP only, it displayed high preference for the synthesis of cGAMP when both substrates were present. Moreover, in extracts from *V. cholerae* mainly cGAMP was detected. Of note, DncV showed no homology to previously known dinucleotide cyclases, yet rather resembled eukaryotic 2′-5′-oligoadenylate synthetases and poly(A) polymerases, which are part of the nucleotidyl transferase superfamily. In line with this observation, it later turned out that DncV synthesizes cGAMP in a process that is distinct from prokaryotic cyclic dinucleotide synthases, which function as obligate oligomers. Indeed, similar to OAS enzymes and cGAS (see below) DncV harbors a single catalytic site that catalyzes the formation of a cyclic dinucleotide in a two-step process (Kranzusch et al. 2014). The exact mechanistic role for *V. cholerae* cGAMP is not clear; yet it has been suggested that the hybrid cGAMP molecule can either function via the c-di-GMP and c-di-AMP pathways or initiate a completely new biological process. At the same time, our knowledge about the function, the interacting receptors, or the mechanism of degradation of cGAMP is limited. So far a cGAMP-sensing riboswitch has been identified (Kellenberger et al. 2015), and one study reports proteins carrying an HD-GYP domain that is responsible for degradation of 3′-3′-cGAMP (Gao et al. 2015) (Table 1).

3 Cyclic Dinucleotides in Metazoa

3.1 CDNs Can Activate the Innate Immune System

Despite the extensive knowledge about the synthesis and the role of CDNs in prokaryotes, archaea and more recently in lower eukaryotes, it remained unclear whether similar molecules would exist or be detected in the metazoan system. The first indication that cyclic dinucleotides were indeed sensed by metazoan cells came from studies using mice that were treated with c-di-GMP in the context of *Staphylococcus aureus* infection. While initially intended to act as a direct antimicrobial substance (Karaolis et al. 2005), it turned out that c-di-GMP led to a pronounced activation of the innate immune system in this setting. To this end, a number of in vitro and in vivo studies in mice showed that c-di-GMP application led to a potent activation of cells of the innate immune system, resulting in considerable pro-inflammatory gene expression (cytokine, chemokine expression, induction of co-stimulatory molecules) and subsequent activation of cells of the adaptive immune system. In fact, applying c-di-GMP as an adjuvant in a prophylactic vaccination setting resulted in a dramatic increase in antibody titers (Karaolis et al. 2007a) that were protective in the context of various models of bacterial infection (Hu et al. 2009; Ogunniyi et al. 2008). At the same time, prophylactic, monotherapeutic application of c-di-GMP prior to bacterial inoculation in a lung infection model resulted in a marked decrease in bacterial burden, most likely due to the nonspecific activation of innate immune cells (Karaolis et al. 2007b). Altogether, these studies implicated that c-di-GMP could serve as a classical

microbe associated molecular pattern (MAMP) molecule to the innate immune system; yet the respective pattern recognition receptor (PRR) had remained elusive. The first hint came from a subsequent study by the group of Vance and coworkers, showing that c-di-GMP triggered a cytosolic PRR that initiated antiviral and pro-inflammatory gene expression in a TBK1-IRF3-dependent fashion, while known PRR pathways (toll-like receptors and RIG-I-like receptors) could be ruled out (McWhirter et al. 2009). However, in the course of these studies it was noted that c-di-GMP recognition triggered a transcriptional program that was similar to the cytosolic DNA recognition pathway, a signaling cascade that had also remained unresolved at that time. First evidence that cytosolic recognition of bacterial cyclic dinucleotides played an important role in microbial detection came from studies with *L. monocytogenes*, a gram-positive bacterium that replicates in the cytosol of host cells. Here it was shown that *Listeria*-mediated induction of type I IFN production closely correlated with its ability to produce and secrete c-di-AMP (Woodward et al. 2010). As c-di-AMP triggers immune-stimulatory effects, also c-di-AMP has been shown to work as an adjuvant in vaccination (Sanchez et al. 2014; Ebensen et al. 2011).

3.2 STING Is the Receptor for Prokaryotic CDNs

The important clue regarding the identity of the sensor for the prokaryotic CDNs resulted from a forward genetic mutagenesis screen in mice that were tested for their responsiveness towards c-di-GMP (Sauer et al. 2011). In fact, a nonsense mutation in the ER-resident protein STING (Stimulator of interferon gene protein) completely abolished the in vitro response to c-di-GMP (Sauer et al. 2011). Analogous findings were observed for c-di-AMP in STING-deficient animals (Jin et al. 2011). Of note, STING was not an unknown player in innate immunity. In fact, just a few years prior to its implication in c-di-GMP recognition, STING had been discovered as an essential component in cytosolic DNA detection (Ishikawa and Barber 2008; Ishikawa et al. 2009). However, its exact role as a putative receptor or an intermediate signaling molecule in this pathway had remained unclear. Further analysis however revealed that both c-di-GMP and c-di-AMP could directly interact with STING (Burdette et al. 2011). The specificity for this interaction turned out to be very high as demonstrated by the absence of binding with AMP, GMP, or cAMP (Burdette and Vance 2013).

STING is tethered to the ER through four consecutive N-terminal transmembrane domains, whereas its C-terminal ligand-binding domain faces the cytoplasmic lumen. Two STING molecules form a preformed homodimer through their C-terminal domains forming a binding pocket for one cyclic dinucleotide molecule. Upon binding with its ligand, STING undergoes a conformational switch that results in the reorganization of its lid structure (Cai et al. 2014). As a consequence, its long unstructured carboxy-terminal tail opens up, permitting the recruitment and autophosphorylation of TBK1 and the subsequent activation of IRF3. At the same time, STING activation results in the activation of STAT6 and NF-κB. These

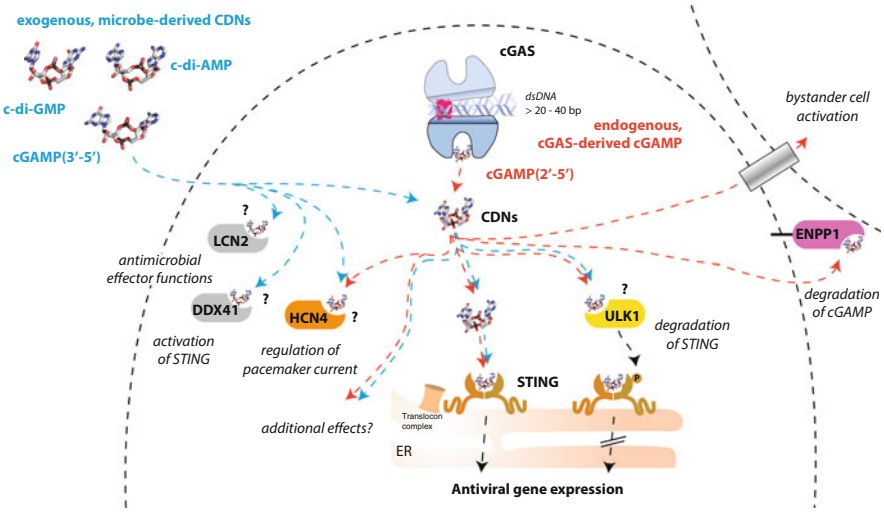

Fig. 2 Signaling cascades triggered in mammalian cells by exogenous or endogenous CDNs. A schematic overview of a mammalian cell is given. CDNs of exogenous (*blue color*) or endogenous sources (*red color*) can engage a number of different pathways within the cell: Most importantly, CDNs engage the ER-resident PRR STING, which results in the induction of antiviral gene expression. Apart from STING, several other target proteins have been described to bind CDNs, thereby affecting various pathways: antimicrobial effector functions (LCN2), activation of STING (DDX41), regulation of pacemaker currents within the heart (HCN4), or degradation of STING (ULK1) or cGAMP (ENPP1). Moreover, beyond its function of inducing cell-autonomous immune responses, cGAMP can also travel through gap junctions to trigger STING activation within neighboring cells

transcription factors travel to the nucleus to transactivate their responsive promoters, thereby inducing antiviral and pro-inflammatory gene expression (Fig. 2). Of note, once activated STING exits the ER in direction of the ER-Golgi intermediate compartment (ERGIC) and Golgi complex, while later it is found in punctate structures. TBK1 recruitment occurs as early as STING enters the ERGIC; yet the exact spatiotemporal relations are not entirely clear (Dobbs et al. 2015).

3.3 The cGAS-STING Axis

Having identified STING as the direct receptor for cyclic dinucleotides, the question remained how cytosolic DNA recognition was also dependent on STING. This conundrum was elegantly solved by the group of Chen and coworkers, who identified a cytoplasmic nucleotidyltransferase they named cGAS (cyclic GMP-AMP synthase) that was directly activated by DNA to generate the hybrid cyclic dinucleotide molecule cGAMP (Wu et al. 2013; Sun et al. 2013). cGAS displays a two-lobed structural fold, in which a positively charged platform binds to dsDNA in a sequence-independent manner. One key element of this platform is the

so-called zinc thumb protrusion, which ensures that cGAS can only bind to DNA and not to RNA (Hornung et al. 2014). Upon binding of dsDNA, cGAS undergoes a conformational switch, which in turn initiates its catalytic activity. Analogous to its structural homologue, the *V. cholerae* DncV enzyme, cGAS catalyzes cGAMP in two sequential steps, wherein the same catalytic center carries out both nucleotidyltransferase reactions. Nevertheless, two cGAS molecules form a homodimer with two dsDNA molecules (2:2 ratio), while the catalytic center faces outward. Although it was initially thought that cGAS produced a hybrid cyclic dinucleotide molecule with two 3′-5′-phosphodiester linkages, three independent routes of investigation clarified that the metazoan cGAMP differed from prokaryotic cGAMP in a small, but important aspect. To this end, cGAS-derived cGAMP was found to harbor mixed phosphodiester linkages between its ring-forming ribose molecules: a noncanonical 2′-5′ phosphodiester linkage between the GMP and the AMP and a 3′-5′ phosphodiester linkage between AMP and GMP (>Gp(2′-5′)Ap(3′-5′)> or cGAMP(2′-5′)) (Ablasser et al. 2013a; Diner et al. 2013; Gao et al. 2013a) (Fig. 1). This difference in phosphodiester linkages has fundamental consequences for the activity of metazoan cGAMP(2′-5′). In fact human STING, in contrast to murine STING, is far more sensitive to cGAMP(2′-5′) as compared to its prokaryotic counterpart (Ablasser et al. 2013a; Diner et al. 2013). This species specificity in cyclic dinucleotide recognition is attributable to subtle structural differences in those proteins (Zhang et al. 2013). Altogether, these studies established a new type of signaling cascade, in which a cyclic dinucleotide functions as a second messenger to activate a PRR.

3.4 Evolution of the cGAS-STING Axis

Evolutionary analyses suggest that cGAS and STING can be traced back to early metazoan evolution. Indeed, cGAS and STING are already found in *choanoflagellates*, unicellular eukaryotic organisms with the closest evolutionary relationship to metazoans (Wu et al. 2014). Moreover, functional analyses suggest that the cGAS-CDN-STING signaling is already operational as early as in the *Cnidaria* phylum (Kranzusch et al. 2015), predating the development of the type I IFN system, which arose in the vertebrate lineage. Of note, in contrast to its vertebrate counterpart, anemone cGAS generates a 3′-5′-linked cGAMP molecule, and furthermore, it does not respond to dsDNA (Kranzusch et al. 2015). Indeed, in silico analyses suggest that dsDNA recognition, which requires the Zn thumb protrusion in the positively charged spine region of the two-lobe cGAS structure, only evolved in the early vertebrate lineage (Wu et al. 2014). Altogether, these observations suggest that CDN signaling evolved in early metazoan evolution prior to its function in dsDNA recognition in the context of innate immunity.

3.5 cGAS-STING Signaling in Antimicrobial Defense

Soon after its discovery, several studies outlined the important contribution of cGAS and the cGAMP-STING axis in the innate immune response towards microbes. To this end, several in vitro and in vivo studies have highlighted a role for cGAS in the recognition of DNA viruses, cytosolic bacteria, and also parasites (Gao et al. 2013b; Zhang et al. 2014; Hansen et al. 2014; Schoggins et al. 2014; Wassermann et al. 2015; Collins et al. 2015; Watson et al. 2015; Majumdar et al. 2015). A number of studies suggest that cGAS and STING play nonredundant roles in the induction of pro-inflammatory gene expression by these pathogens, which would indicate that cGAMP signaling only engages STING as its receptor. In fact, comparing cGAS to STING knockout cells in the context of various microbial pathogen infection models indicates that deficiencies in these proteins create a complete phenocopy (Gao et al. 2013b; Watson et al. 2015). Interestingly, even a number of bacteria for which cyclic dinucleotide synthesis is known (e.g., *Legionella*, *Mycobacteria*) are detected in a cGAS-STING-dependent fashion (Zhang et al. 2014; Watson et al. 2015). In contrast, other microbes (e.g., Listeria) are directly sensed by STING, without a requirement for cGAS; yet there seems to be a cell type specificity in the usage of cGAS (Watson et al. 2015). Whether STING plays a role in DNA recognition beyond cGAS and beyond its function as a cyclic dinucleotide receptor awaits further exploration. Of note a number of DNA receptors have been postulated to operate upstream of STING (IFI16, DDX41, RAD50, MRE11); yet these models require additional genetic validation (Hornung 2014a, b). Indeed, the fact that cGAS-deficient cells display a complete lack of dsDNA-triggered STING activation in various cell types argues against existence of an additional DNA receptor upstream of STING. However, it is possible that the previously described, alternative DNA sensors facilitate cGAS-STING signaling under certain conditions. Nevertheless, it is noteworthy that a recent study has provided genetic proof for a cGAS-independent activation of STING in the context of DNA vaccination (Suschak et al. 2016). Future studies will be required to address whether STING is activated in a CDN-independent manner under these circumstances or even by an alternative CDN (Burdette and Vance 2013). Regarding alternative sources of CDNs, there is currently no indication of cyclic dinucleotide synthases beyond cGAS in the mammalian system (Wu et al. 2014). While there are a number of Mab-21 domain-containing proteins that have not fully been explored in the human system, their role in activating STING appears rather unlikely. However, it is possible that alternative stimulatory conditions exist, in which CDNs generated independently of cGAS are playing a role.

3.6 Role of the cGAS-STING Axis in Sterile Inflammation

The detection of intracellular DNA from infecting bacteria, viruses, or protozoa and the resulting production of pro-inflammatory cytokines and type I IFNs assist in the clearance of the pathogen by the innate immune cells. However, host cells themselves can be a source of DNA. Phagocytosis of apoptotic or necrotic material or

mislocalization of endogenous material can result in the cytoplasmic recognition of DNA and thus activation of the downstream signaling pathways (Ablasser et al. 2013b). In this regard it has to be noted that cytoplasmic DNA sensors such as cGAS do not discriminate self from non-self nucleic acids and detect DNA just due to its double-stranded conformation (Roers et al. 2016). Under steady-state conditions, the erroneous detection of cytoplasmic DNA by the cGAS-STING axis and other pathways is counteracted by a number of enzymes that degrade nucleic acids in different compartments (e.g., TREX1/DNase III in the cytoplasm, DNase II in the lysosome, DNase I in the extracellular space). Of note, this nuclease safety net is nonredundant. To this effect, absence or dysfunctionality of already one of these enzymes already results in continuous activation of nucleic acid-sensing PRRs and thereby severe inflammation (Crow and Manel 2015). Along this line, deficiency of TREX1 causes the accumulation of DNA in the cytoplasm and thereby triggers a constant activation of the cGAS-STING axis (Ablasser et al. 2014). Another example for erroneous recognition of self-DNA is the detection of mitochondrial DNA that can gain access to the cytoplasm in the context of mitochondrial stress (West et al. 2015) or disintegration (Rongvaux et al. 2014; White et al. 2014). Again appearance of self-DNA in the cytoplasm results in the activation of the cGAS-STING axis.

Another, yet "beneficial" example for the involvement of cGAMP in the induction of a sterile inflammatory response can be observed within the tumor microenvironment. Spontaneous, cytotoxic CD8+ T-cell responses targeting tumor cells have been documented in many different tumor entities, whereas a strong correlation of tumor-intrinsic type I IFN production and T-cell activity could be observed. Subsequent studies revealed that tumor-infiltrating dendritic cells constituted the major source and also receptive population of this type I IFN response (Corrales and Gajewski 2015). Moreover, it was uncovered that the cGAS-STING pathway plays a predominant role in driving this type I IFN response, at least for the tumor models tested. In this respect, it was shown that DNA derived from dying tumor cells was taken up by tumor-infiltrating dendritic cells, which then in turn engaged the cGAS-STING axis to drive antigen-specific immune responses (Woo et al. 2014; Deng et al. 2014; Klarquist et al. 2014). Altogether, these results imply that cGAS-STING signaling within tumor tissues is able to drive potent antitumor immune responses, thereby providing a rationale to employ CDNs as STING ligands for antitumor therapy (see below).

3.7 cGAMP Activity Beyond Cell-Autonomous Immunity

Beyond its role in cell-autonomous innate immune signaling, cGAMP is also subject to horizontal cell-to-cell transfer via intercellular gap junctions (Ablasser et al. 2013c). By this in trans signaling mechanism cell intrinsic immunity is very rapidly communicated to neighboring cells in a transcription-independent manner. From the host perspective, this mechanism provides several key advantages. Avoiding the detour and the delay of the paracrine type I IFN system, antiviral

immunity is rapidly communicated between neighboring cells by physically linking their signaling cascades. Moreover, a hallmark of virus-infected cells is the suppression of certain biosynthetic pathways (e.g., translational inhibition) to slow or alter viral propagation. As such cGAMP transfer still enables cells that have been compromised by the infection to fight microbial propagation by employing neighboring cells as their extended and amplifying signaling output. At the same time, this mechanism also implies that cGAS-dependent STING activation can also extend to cells that do not express cGAS, yet are competent for STING. Indeed, comparing the expression profile of cGAS and STING reveals that these genes are not strictly co-expressed, which supports the argument for an intercellular cooperation of these two proteins beyond cell boundaries. Nevertheless, additional studies are required to judge the physiological relevance of this signaling route.

More recently two groups identified another route taken by cGAMP for its transmission to adjacent cells (Bridgeman et al. 2015; Gentili et al. 2015). Here it was revealed that upon infection with viruses and the detection of the viral DNA by cGAS, the synthesized cGAMP can "piggyback" inside the viral particles, which when they infect other cells also transfer the pre-synthesized cGAMP. Thereby encapsulated cGAMP provides a preinfection warning signal that promotes the upregulation of antiviral genes in these cells. Again, additional experimental proof is required to validate the functional relevance of this pathway.

3.8 CDN Activities Beyond STING

Another intriguing question relevant for the mammalian system is whether cyclic dinucleotides exert biological effects beyond their recognition by STING. In contrast to the prokaryotic world, where cyclic dinucleotides target multiple receptors within the same cell, current evidence suggests that CDN signaling is far more restricted in the metazoan system. This is already documented by the fact that the CDN receptor STING and its CDN-binding domain are unique within the metazoan genome. In prokaryotes, on the contrary, a large number of genes regulate CDN synthesis, detection, and degradation (e.g., in *V. cholerae* encodes for at least 72 DGC and PDE proteins (Danilchanka and Mekalanos 2013)). Nevertheless, since the delineation of the cGAS-STING axis a few reports have suggested that additional functions beyond this strict hardwiring exist.

Prior to the discovery of cGAS, a study had reported that c-di-GMP and cyclic-di-AMP also directly bind to the cytosolic helicase DDX41 (Parvatiyar et al. 2012). In this setting, CDN-bound DDX41 in turn engaged STING to induce signal transduction, whereas DDX41 involvement was critically required to activate STING (Fig. 2). Up to date, structural data and genetic loss-of-function studies are not available to corroborate this model. To this effect, future studies will be required to substantiate the role of DDX41 in CDN signal transduction.

On the other hand, it has been shown that cGAMP triggers the phosphorylation of STING by activating the autophagy regulator ULK1 (Konno et al. 2013). In this setting both canonical and noncanonical cGAMP inhibited the AMPK signaling

complex that represses ULK1 activity without prior STING engagement. This in turn resulted in ULK1-dependent phosphorylation and degradation of STING. Altogether, these results suggest that cGAMP directly engages the AMPK pathway. It will be interesting to further explore the role of this connection to energy homeostasis; moreover it should be of interest to elucidate the direct receptor of cGAMP in this cascade.

Another unexpected target of cyclic dinucleotides in the mammalian system was found to be an ion channel that regulates conductance in cardiac pacemaker myocytes (Lolicato et al. 2014). HCN4 is a potassium sodium hyperpolarization-activated ion channel that is known to be regulated by intracellular cAMP and cGMP. cNMP-mediated regulation is achieved by a cytosolic cyclic nucleotide-binding domain (CNBD) that allosterically modulates the conductance of the ion channel. In the course of crystallization studies a second cNMP-binding pocket was found next to the canonical cNMP pocket. This binding pocket accommodated canonical as well as noncanonical cyclic dinucleotides and occupancy of this pocket counteracted the effect of cAMP. This resulted in the inhibition of the cAMP-mediated activation of the channel, thereby reducing the heart rate (Fig. 2). Additional studies are needed to confirm the physiological role of this rather unexpected link, yet in keeping with the prokaryotic world this appears like a plausible regulatory connection. In fact, as described above, a number of ion channels are known as important regulatory targets in the case of c-di-AMP.

In addition, a recent study employed an inverse docking approach and identified the antibacterial component Siderocalin (LCN2) to directly bind c-di-GMP (Li et al. 2015) (Fig. 2). CDN-bound LCN2 was unable to bind bacterial ferric siderophores and thereby lost its antimicrobial activity. Of note, c-di-AMP or endogenous cGAMP did not bind to LCN2, which would render this regulatory connection specific to c-di-GMP.

Finally, it has been noted that cGAS exerts biological effects independently of its catalytic function (Liang et al. 2014). Upon DNA stimulation, cGAS has been shown to interact with Beclin-1, resulting in its activation and thus initiation of autophagy without the requirement for STING. At the same time, this interaction negatively regulates cGAS activity. While these regulatory circuits appear to function independently of CDN synthesis, it is important to keep them in mind when evaluating cGAS-dependent, yet STING-independent, functions, as they have for example been observed in the context of mycobacterial infection (Collins et al. 2015).

3.9 Degradation of cGAMP

Every innate immune response has to be terminated to avoid excessive stimulation of antimicrobial defense mechanisms, which can be just as detrimental to the host as the microbial insult itself. As outlined above, in prokaryotes a large number of phosphodiesterases have been shown to degrade CDNs. In a search for a cGAMP-degrading enzyme in human cells, Li and colleagues undertook a biochemical

approach by characterizing different subcellular fractions for cGAMP-degrading activity. Doing so, they made the surprising discovery that the membrane, but not the cytoplasmic fraction, led to cGAMP degradation. Subsequent studies revealed that the ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP1) was the dominant cGAMP-degrading enzyme (Li et al. 2014). Tissue from Enpp1 knockout mice displayed attenuated cGAMP-degrading activity. In line with this finding, a hydrolysis-resistant bisphosphothioate analog of cGAMP (cG^sA^sMP) could not only bind to STING but also induce a stronger type I IFN response. Given the fact that the catalytic activity of ENPP1 faces the extracellular space, these data suggest that cGAMP activity is not counteracted within the cytoplasm (Table 1, Fig. 2).

4 Therapeutic Application of Cyclic Dinucleotides

4.1 Adjuvants for Prophylactic Vaccines

As outlined above, early studies exploring the therapeutic effects of c-di-GMP focused on its direct antimicrobial activities (Karaolis et al. 2005). Subsequent studies, however, have revealed that the beneficial effects of c-di-GMP application in vivo were mainly attributable to immunogenic effects that were directly elicited within the host (Karaolis et al. 2007b). In line with its immunostimulatory activities, vaccination studies revealed that CDNs or modified versions thereof could be employed as prophylactic vaccine adjuvants to induce antigen-specific immune responses after systemic and also mucosal vaccination (Ogunniyi et al. 2008; Ebensen et al. 2007, 2011; Chen et al. 2010; Libanova et al. 2010). In these studies CDN application could trigger the formation of protective antibody titers as well as the formation of a potent antigen-specific CD4+ and CD8+ T cell response (for a review see (Dubensky et al. 2013)). Future studies will need to be conducted to explore whether CDNs will qualify as safe adjuvants for prophylactic vaccines in the human system.

4.2 Adjuvants for Therapeutic Vaccines

Given the fact that immunogenic tumors display a beneficial engagement of the cGAS-STING axis, excitement was spurred to therapeutically engage STING within tumor tissues. In fact, the successful targetability of the STING pathway was already validated in the “pre-STING era” in murine tumor models. To this end, the small-molecule drugs flavone acetic acid (FAA) and 5,6-dimethylxanthenone-4-acetic acid (DMXAA) showed impressive activities in various solid tumor models in rodents (Pang et al. 1998; Hornung et al. 1988; Ching and Baguley 1987). Similarly, the structurally related compound 10-carboxymethyl-9-acridanone (CMA) displayed potent antiviral and antitumor activities in mice (Kramer et al. 1976; Storch et al. 1986). However, FAA, DMXAA, and CMA

turned out to be species-specific agonists for murine, but not human STING (Cavlar et al. 2013; Conlon et al. 2013; Kim et al. 2013), which provided a mechanistic explanation for their failure to translate into clinical application. Following its discovery as a natural STING ligand, c-di-GMP was also studied as an immunostimulatory compound in a murine tumor model (Chandra et al. 2014). Here, c-di-GMP showed potent therapeutic antitumor activity in a model of metastatic breast cancer when applied in conjunction with a tumor-specific antigen, but also when applied at high doses on its own. Given their lower and genotype-specific efficacy towards human STING, CDNs with two 3′-5′-phosphodiester linkages, such as c-di-GMP, might not be ideal candidates for future clinical applications. As such, a more recent study developed CDNs with a mixed 2′-5′/3′-5′-dithio linkage. In contrast to bacterial CDNs, these molecules showed agonistic activity towards all major human STING variants, apart from their enhanced half-life. Furthermore, in vivo, these CDNs displayed potent antitumor activity when applied intratumorally (Corrales et al. 2015).

5 Conclusions

From the earliest reports identifying the presence of CDNs in prokaryotes to the recent discovery of cGAMP within the eukaryotes, significant progress has been made in our understanding of the both the biochemical properties and the functional relevance of this unique class of nucleotide second messengers. In the vertebrate system CDNs of both exogenous and endogenous sources engage the ER-resident PRR STING to trigger antiviral immunity. At the same time, current research suggests that additional, STING-independent pathways are activated or modulated by CDNs, yet more research is required to substantiate these concepts.

Studies in the murine system suggest that targeting STING using natural or modified CDNs evokes potent innate immune responses in vivo. As such, CDNs provided potent adjuvanticity when combined with antigens in a prophylactic vaccination setting. Moreover, murine tumor models suggest that activating STING using CDNs might prove a successful strategy to trigger antitumor immunity. However, as promising as these results are, the past has told us to be cautious about extrapolating murine tumor studies to the human system. Apart from that, therapeutic approaches aimed at blocking cGAS-mediated CDN synthesis or its activation of STING in the context of sterile inflammatory conditions might be just as promising.

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Mass Spectrometric Analysis of Non-canonical Cyclic Nucleotides

Heike Bähre and Volkhard Kaever

Contents

Abstract

Contemporary investigations regarding the (patho)physiological roles of the non-canonical cyclic nucleoside monophosphates (cNMP) cytidine 3′,5′-cyclic monophosphate (cCMP) and uridine 3′,5′-cyclic monophosphate (cUMP) have

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been hampered by the lack of highly specific and sensitive analytic methods for these analytes. In addition, the existence of 2′,3′-cNMP besides 3′,5′-cNMP has been described recently. HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) is the method of choice for identification and quantification of low-molecular weight endogenous metabolites. In this chapter, recommendations for an HPLC-MS/MS method for 3′,5′- and 2′,3′-cNMP are summarized.

Keywords
3′,5′-cNMPs • 2′,3′-cNMPs • Liquid chromatography • Mass spectrometry

1 Introduction

The well-known cyclic nucleoside monophosphates cAMP and cGMP play key regulatory roles as signaling molecules in all kingdoms of life (Gancedo 2013; Schlossmann and Schinner 2012). In contrast, the non-canonical cyclic nucleotides cCMP and cUMP also occur in vivo (Bähre et al. 2015), but elucidation of their (patho)physiological roles is just at the beginning (Seifert et al. 2015). The availability of reliable detection methods for all cNMP is an absolute requirement for further research in this field. Numerous detection methods for cAMP and cGMP have been described (Berrera et al. 2008; Schmidt 2009) including high performance liquid chromatography (HPLC) with UV or fluorescence detection, immunoassays such as RIA or ELISA, and fluorescence resonance energy transfer (FRET) techniques (Kalia et al. 2013). HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) is a powerful tool for the analysis of low-molecular weight signaling molecules (Roux et al. 2011). It is characterized by high sensitivity and selectivity and allows simultaneous identification and quantification of canonical as well as non-canonical cNMP. However, due to their low concentrations in biological fluids as well as rapid metabolism, analysis of cNMP is a highly challenging analytical task. Special care has to be taken regarding the initial sample preparation steps, the robustness of the HPLC method applied, and reliable MS/MS recordings. In this case, HPLC-MS/MS with inclusion of isotope-labeled internal standards and assessment of specific quantifier and qualifier mass transitions is advised. We here present two examples of cNMP analyses by HPLC-MS/MS in biological matrices, i.e., murine tissues and human urine.

2 High Performance Liquid Chromatography-Coupled Mass Spectrometry (HPLC-MS/MS)

First step of the analysis of a sample containing a mixture of small molecules (e.g., cNMP) is the liquid chromatographic separation step. For cyclic nucleotides separation is usually performed on a C18 HPLC column (Zhang et al. 2009; Beste et al. 2012; Jia et al. 2014; Bähre and Kaever 2014) or on porous graphitic carbon

(Martens-Lobenhoffer et al. 2010; Pabst et al. 2010) using reversed phase chromatography. Furthermore, Goutier et al. (2010) performed chromatographic separation of cAMP and ATP in hydrophilic interaction liquid chromatography (HILIC) mode. Since in HPLC molecules are separated due to their chemical structures, those molecules with a high structure similarity may show similar retentional behavior and may therefore coelute from the HPLC column. Whereas in UV detection coeluting molecules with similar absorbance maxima cannot be differentiated from each other, mass spectrometry enables the discrimination of even these molecules.

The mixture of molecules coeluting from the HPLC column is often ionized by electrospray ionization (ESI) in the ion source of the mass spectrometer. A high voltage is applied and induces droplets containing either positive or negative charged ions. Although cNMP were mainly detected in positive ionization mode, in some studies detection in negative ionization mode is reported (Zhang et al. 2009; van Damme et al. 2012). Due to heat-induced desolvation the droplets shrink until the repulsive force inside the droplets becomes too strong resulting in a Coulomb explosion and finally in gaseous ionized molecules. These molecules are accelerated towards the first quadrupole (Q1) of the mass spectrometer where they are separated according to their mass to charge ratio (*m*/*z*) and only those molecules with a preset *m*/*z*-value will be able to pass the quadrupole on a stable trajectory (Fig. 1). Ions with a differing *m*/*z*-value will be discharged at the rods of the quadrupole and will not pass the quadrupole. Normally we expect all ions to have different *m*/*z*-ratios and only the one ion we are interested in will pass Q1 resulting in an unequivocal signal in a chromatogram. However, it is possible that the *m*/*z*-ratio of at least one coeluting molecule is very similar to the desired analyte. Tandem mass spectrometry (MS/MS) enables the discrimination even between critical molecules by fragmentation. After passing the first quadrupole the so-called precursor ions enter the collision cell (q2) of the mass spectrometer where the fragmentation takes place. The precursor ion collides with an inert collision gas (usually nitrogen or argon) and will dissociate in analyte specific fragment ions. Cyclic nucleotides are usually fragmented by a cleavage of the

Fig. 1 Theoretical background of HPLC-coupled tandem mass spectrometry (HPLC-MS/MS). After chromatographic separation structurally similar molecules ("A," "B," and "C," with "A" being the desired analyte) may coelute from the HPLC column and are ionized in the source of the mass spectrometer at the same time. These compounds enter the first quadrupole (Q1) where ions are selected regarding their *m*/*z*-values. And only those ions with a distinct *m*/*z*-value are able to pass this quadrupole. But some compounds (e.g., "A" and "B") may have very similar *m*/*z*-values and therefore, cannot be discriminated. Both ions enter the collision cell (q2) and will be fragmented ("a_1–a_3" and "b_1–b_3"). To discriminate between molecule A and B a specific fragment of A (e.g., fragment a_1) is selected in the third quadrupole (Q3) and finally passes through to the detector. Other precursor or fragment ions can be selected by changing quadrupole parameters, which allows a simultaneous detection of numerous analytes in one analytical run

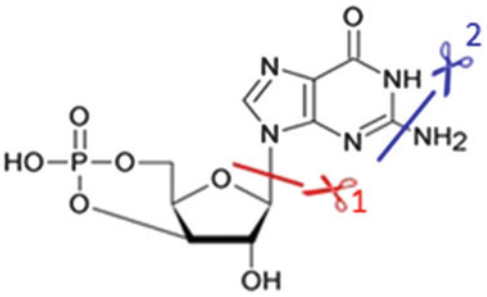

Fig. 2 Structure and fragmentation of cGMP. The dominant fragment *m*/*z* 152 results from the cleavage of the bond between the nucleobase and the ribose residue (cleavage 1) and is usually used as quantifier. A further loss of the ammonia group results in a second fragment (*m*/*z* 136; cleavage 2), which can function as identifier

glycosidic linkage of the nucleobase and the ribose residue resulting in the base as main fragment (Fig. 2). Additional fragments result, e.g., from further fragmentation of the base or are a result of its rearrangement (Bähre and Kaever 2014).

All created fragments are accelerated and reach the last quadrupole (Q3), where they are sorted again according to their mass to charge ratios. Only selected fragments are enabled to pass through to the detector. As a consequence, the resulting chromatogram only shows signals of the mass transitions of selected analytes. Matrix components or other small molecules with differing mass to charge ratios either of precursor and/or fragment ions are ignored. As a result, an MS/MS-based chromatogram shows a significantly reduced amount of signals in comparison to UV-based methods. This fascilitates the unequivocal determination of cNMPs especially when cNMP analysis in biological matrices like tissues is required.

3 Quality Criteria in cNMP Identification by HPLC-MS/MS

Although HPLC-MS/MS is characterized by high sensitivity and, compared to HPLC-UV, high selectivity, analysis may be influenced by matrix components in various ways (Tylor 2005). If a matrix component has (nearly) the same mass transition like a desired cNMP, the resolution of the quadrupoles may not be high enough to discriminate between the matrix component and the cNMP of interest. This can lead to signals in the chromatogram that do not belong to the cNMP and therefore may falsify the result. To ensure a signal really reflects the analyte certain quality criteria should be met.

3.1 Absolute Retention Time

Coupling of tandem mass spectrometry to chromatography allows the discrimination of compounds not only based on their mass transitions but, moreover, based on

their retention times on an HPLC column. In reversed phase chromatography, 3′,5′-cyclic nucleotides usually elute in the following order: cXMP, cCMP, cUMP, cIMP, cGMP, cTMP, and cAMP (Fig. 3), with definite retention times depending on the used chromatographic system (e.g., column material and length, flow rate, solvent system, etc.). By knowledge of the retention times of cNMP standards yet "unknown" signals can be assigned to a cNMP.

Not only matrix components but also cNMPs themselves can cause additional signals in a chromatogram. The *m*/*z*-values of the cNMP pairs cAMP/cIMP, cCMP/cUMP and cGMP/cXMP only differ in one Dalton (Table 1). As a consequence, the natural isotopes [(M+1)+H]+ of the lighter cNMPs (cAMP, cUMP, and cGMP)

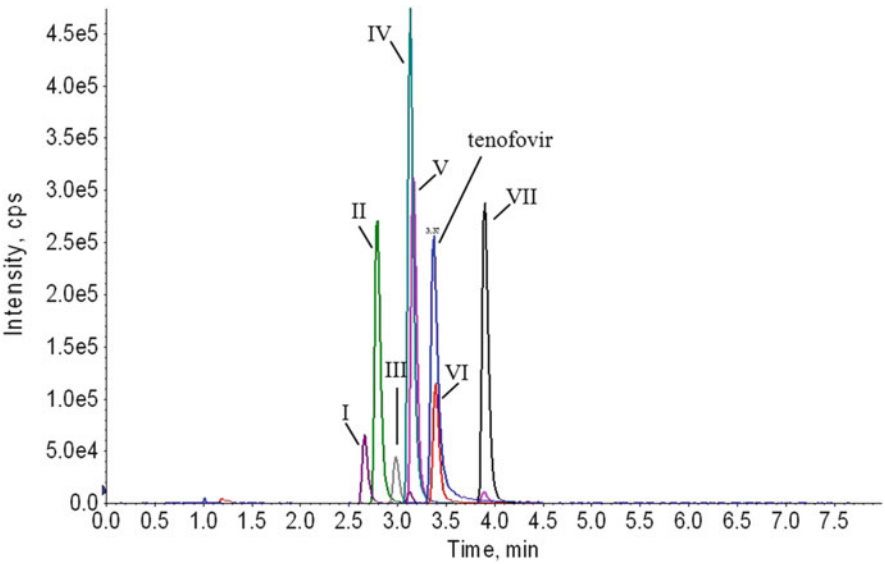

Fig. 3 Typical cNMP chromatogram achieved by reversed phase chromatography. Shown are the quantifier mass transitions of the seven cNMPs cXMP (*I*), cCMP (*II*), cUMP (*III*), cGMP (*IV*), cIMP (*V*), cTMP (*VI*), and cAMP (*VII*), and the signal of the internal standard tenofovir

Table 1 Masses of precursor ions and fragment ions of cyclic nucleotides found in tandem mass spectrometry using positive ionization mode

Cyclic nucleotide	Neutral mass (Da)	Precursor ion (*m*/*z*)	Fragment ion 1 (quantifier) (*m*/*z*)	Fragment ion 2 (identifier) (*m*/*z*)
cAMP	329	330	136	119
cCMP	305	306	112	95
cGMP	345	346	152	135
cIMP	330	331	137	110
cTMP	304	305	127	81
cUMP	305	306	97	113
cXMP	346	347	153	136

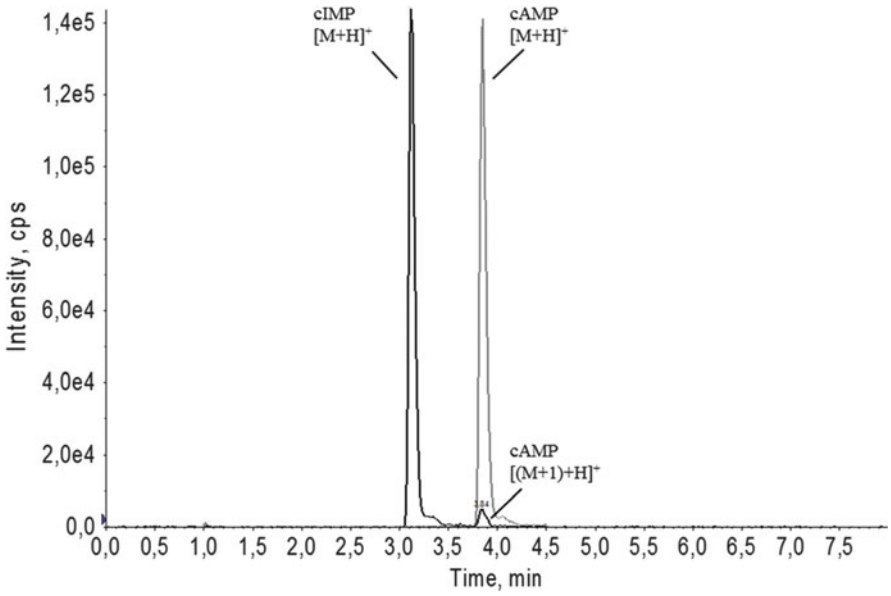

Fig. 4 Chromatogram of the monoisotopic ions of cIMP ($[M+H]^+$; *m/z*: 331 → 137) and cAMP ($[M+H]^+$; *m/z*: 330 → 136) and of the cAMP isotope ($[(M+1)+1]^+$; *m/z*: 337 → 137). Thus, a baseline separation of cIMP and cAMP is essential to ensure that the $[(M+1)+H]^+$ signal of cAMP will not falsify the result for cIMP, especially for high cAMP and low cIMP concentration ranges

show mass transitions with the same *m/z*-values like the corresponding heavier monoisotopic cNMPs (cIMP, cCMP, and cXMP, respectively). This results in a chromatogram, in which the [(M+1)+H]+ peak of, e.g., cAMP appears as (monoisotopic) cIMP (Fig. 4) at the retention time of cAMP. Without a baseline separation of the cNMP pairs named above and the knowledge of their absolute retention times on the HPLC column, the isotope peaks [(M+1)+H]+ of cAMP, cUMP, and cGMP may falsify the results for the monoisotopic cyclic nucleotides cIMP, cCMP, and cXMP, respectively. This interference becomes most evident in case of high concentrations of the lighter cNMP (cAMP, cUMP, or cGMP) while only low concentration of the heavier cNMP is expected.

Furthermore, in cNMP analysis it has to be considered, that, in addition to the well-established 3′,5′-cNMP, 2′,3′-cyclic nucleotides do occur in biological samples (Ren et al. 2009; Jackson et al. 2009; van Damme et al. 2012; Bähre and Kaever 2014). Since these metabolites show a high structural similarity to the 3′,5′-cNMP, their precursor and main fragment ions are identical (Fig. 5). Therefore a discrimination between 2′,3′- and 3′,5′-cyclic nucleotides only based on their mass transitions is not possible. As a consequence, a chromatographic baseline separation is an essential criterion for an unequivocal identification of both isobars.

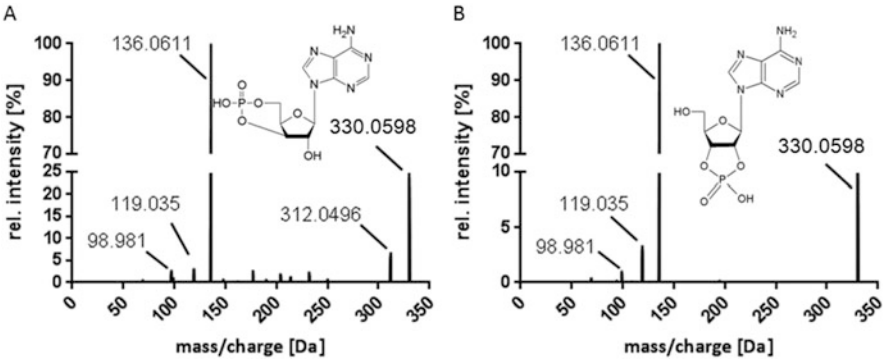

Fig. 5 Fragment spectra of 3′,5′-cAMP (**a**) and 2′,3′-cAMP (**b**)

3.2 Quantifier/Identifier Ratio

In the collision cell (q2) usually more than one fragment ion of a precursor ion is generated and can be selected in the third quadrupole (Q3). The main fragment shows highest intensity and is usually used for quantitation. It is therefore called "quantifier." The detection of additional analyte specific fragments (they are called "identifier" or "qualifier") significantly improves reliability of an analysis, since the ratio between quantifier and identifier transition is an indicator for proper identification and discrimination of an analyte. Only those signals with a specific quantifier/identifier ratio should be used for quantitation (Fig. 6). This procedure is limitated by the intensity of the identifier, which is normally lower than the quantifier intensity. Thus, in low concentration ranges, the identifier signal might be to low or in some cases not even be visible for a ratio calculation.

4 Minimizing Matrix Effects in HPLC-MS/MS

In various samples matrix components do not only result in additional signals but may cause a shift in retention time or influence the ionization efficiency of an analyte. In most cases matrix components lead to a decrease of cNMP ionization efficiency and, as a consequence, to reduced signal intensities. This causes problems especially when only low cNMP concentrations are expected. Furthermore, matrix effects complicate an accurate cNMP quantitation, when there is a differing influence on calibration standards and samples. There are some strategies to minimize the influence of the matrix effects.

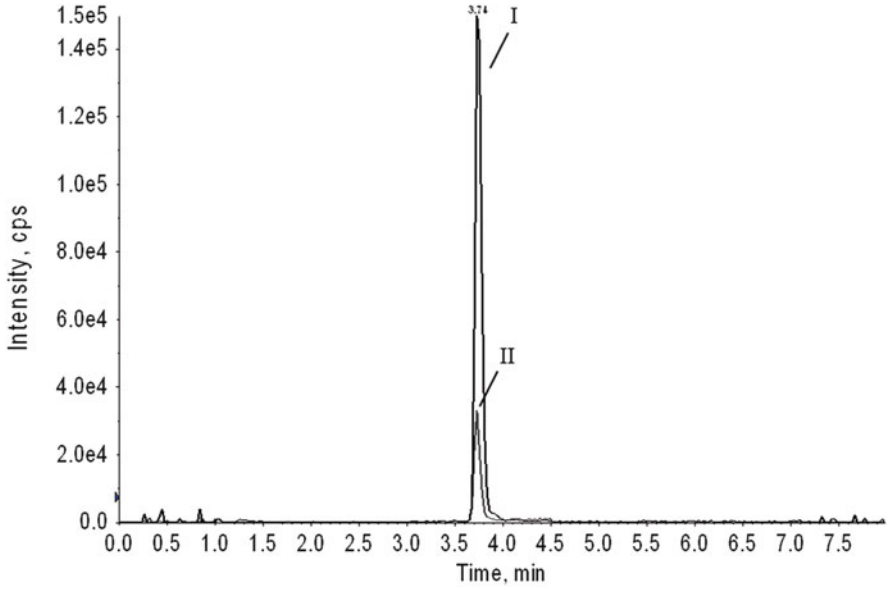

Fig. 6 MS/MS signal of two cAMP mass transitions. The higher signal (signal "I") reflects the AMP quantifier (*m/z* 330 → 136), the lower one (signal "II") the cAMP identifier (*m/z* 330 → 119). Since the ratio of both mass transitions is independent from sample matrix, their peak area ratio can be used as indicator for proper identification of cAMP

4.1 Appropriate Sample Preparation

The most efficient way to minimize matrix effects is the removal of interfering matrix components from the sample as complete as possible before HPLC-MS/MS analysis. In cNMP analysis usually an organic protein precipitation step is performed, which also stops cellular metabolism. Afterwards, the protein content in the sample is removed by centrifugation. To improve HPLC performance, the highly organic supernatant fluid should be evaporated to dryness and subsequently be dissolved in HPLC starting conditions (Zhang et al. 2009; Beste et al. 2012; Bähre and Kaever 2014; Jia et al. 2014).

Another sample preparation procedure using of weak anion exchange solid phase extraction (WAX) suitable to remove matrix components from plasma samples is described by Martens-Lobenhoffer et al. (2010).

4.2 Usage of an Appropriate Matrix for Calibration Standards

By preparing the calibration standards in the same matrix as the samples, the matrix influence on, e.g., retention times and ionization efficiency can be determined and corrected. Since cyclic nucleotides are endogenous metabolites, for most

applications it is not trivial to use exactly the same matrix for calibration standards. In those cases an artificial or surrogate matrix, e.g., bovine serum albumin (BSA), may help to compensate for matrix effects (van der Merbel 2008). For example, Oeckl and Ferger (2012) described the usage of artificial cerebrospinal fluid (aCSF) for the quantification cAMP and cGMP in cerebrospinal fluid (CSF).

Martens-Lobenhoffer et al. (2010) compared the slopes of cGMP calibration curves prepared in plasma versus water, finding a significant intercept for the plasma calibration curve (resulting from endogenous cGMP) but only small differences in the slopes of both curves. As a consequence, the quantification of cGMP even in plasma samples can be performed by an aqueous calibration curve, especially when using an isotope-labeled internal standard (see below). Actually, in most cases the calibration curve is prepared in water when quantifying cNMP in biological matrix (Ren et al. 2009; Zhang et al. 2009; Bähre and Kaever 2014; Dittmar et al. 2015).

4.3 Inclusion of an Internal Standard

An internal standard is strongly recommended in HPLC-MS/MS analytic. Its purpose is on the one hand to evaluate the influence of variations in the sample preparation, on the other hand the minimization of matrix effects. The chemical structure of the internal standard should be as similar as possible to the analyte structure to make sure that the retentional behavior and ionization efficiency are most similar to the analyte ones.

The internal standard plays an essential role in quantitative cNMP analysis. For constructing a calibration curve, the peak area ratio of the analyte and the internal standard is calculated and plotted against the analyte concentration. The peak area ratio from an unknown sample can then be related to the results of the calibration standards, and the cNMP amount in the sample can be determined. In this model the internal standard functions as a normalizing factor by compensating, e.g., losses (of analyte and internal standard) due to the sample preparation procedure and by compensating matrix effects as described above. In cNMP analytic various substances are applied as internal standard (Fig. 7):

- The substitution of the cNMP nucleobase with a bromine results in Br-cNMP derivates, e.g., 8-Br-cAMP, with a similar chemical structure as the native cNMP. Those substances are commercially available, but in HPLC they show longer retention times than native cNMP (Jia et al. 2014)
- Tenofovir is an antiretroviral drug used in treatment of HIV/AIDS and chronic hepatitis B. It is a nucleotide analog, has a nucleobase (adenine) but lacks the ribose sugar. In reversed phase chromatography tenofovir shows similar retentional behavior to cTMP and cGMP and elutes at an intermediate retention time (Fig. 3) (Beste et al. 2012; Bähre and Kaever 2014). Therefore, tenofovir reflects matrix effects for most cyclic nucleotides.

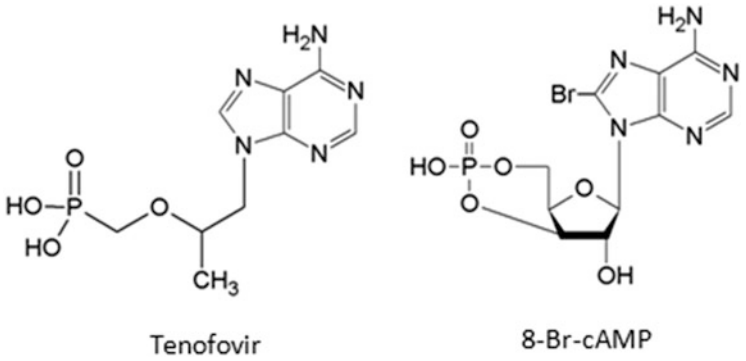

Fig. 7 Chemical structure of tenofovir and 8-bromo-cAMP, two potential internal standards in cNMP analysis

- In some publications the usage of other nucleosides as internal standard is described. For the analysis of rat tissue Ren et al. (2009) found that $^{13}C_{10}$-adenosine is suitable for a reliable quantification of cAMP.

  Furthermore, Göttle et al. (2010) performed several enzyme assays, adding inosine as internal standard to the samples.
- Stable isotope-labeled cyclic nucleotides show, due to the identical chemical structure, the same retention times as "their" unlabeled analyte and reflect therefore the matrix effect best (Fig. 6). The major problem of stable isotope-labeled internal standards is their purity, since the contamination with the unlabeled molecule would falsify the HPLC-MS/MS result especially in low analyte concentration ranges. Furthermore, stable isotope-labeled NMPs are not available and have to be prepared on custom synthesis. However, the enzymatical preparation of $^{13}C_{10}{}^{15}N_5$-cGMP has been described by Zhang et al. (2009) and Martens-Lobenhoffer et al. (2010) using the stable isotope $^{13}C_{10}{}^{15}N_5$-GTP, soluble guanylate cyclase (sGC), and a nitric oxide donor.

This approach can be used for the synthesis of $^{13}C^{15}N$-labeled canonical cNMP, since the required recombinant enzymes are only available for the conversion of labeled GTP to labeled cGMP or labeled ATP to labeled cAMP, respectively. Nevertheless, Göttle et al. (2010) found that edema factor (EF) of *Bacillus anthracis* not only acts as adenylyl cyclase but accepts the substrates CTP, UTP, and GTP as well. Therefore, EF is a universal tool for the synthesis of isotope-labeled canonical and non-canonical cyclic nucleotides (Laue et al. 2014).

In addition to $^{13}C^{15}N$-labeled cyclic nucleotides, Oeckl and Ferger (2012) describe the usage of $^{13}C_5$-cAMP and $^{15}N_5$-cGMP as internal standards for the quantification of cAMP and cGMP in plasma, CSF, and brain tissues, respectively.

5 Examples of cNMP Analyses in Biological Matrices

5.1 Identification of Cyclic Nucleotides in Murine Tissues

When analyzing complex matrices like animal tissues, it becomes obvious that despite a sample preparation procedure, unequivocal analyte identification can be challenging. Figure 8 shows a typical chromatogram of cGMP in a murine bladder sample, in which a quantifier (black trace) and an identifier (red trace) mass transition of cGMP were selected. The resulting chromatogram displays a complex number of signals, with at least three peaks, for which the expected quantifier/identifier ratio for 3′,5′-cGMP is achieved (peak A, B, and C). Only by knowledge of the cGMP retention time in an authentic standard sample, peak C (retention time: 3.1 min) can be assigned to 3′,5′-cGMP, whereas peak A can be identified as 2′,3′-cGMP. The identity of signal B is still unknown.

In this example the advantage and the need of an appropriate chromatographic step become evident. Without that separation step, the discrimination between the signals A, B, and C would not be possible and, as a consequence, the 3′,5′-cGMP amount in this sample would be overestimated.

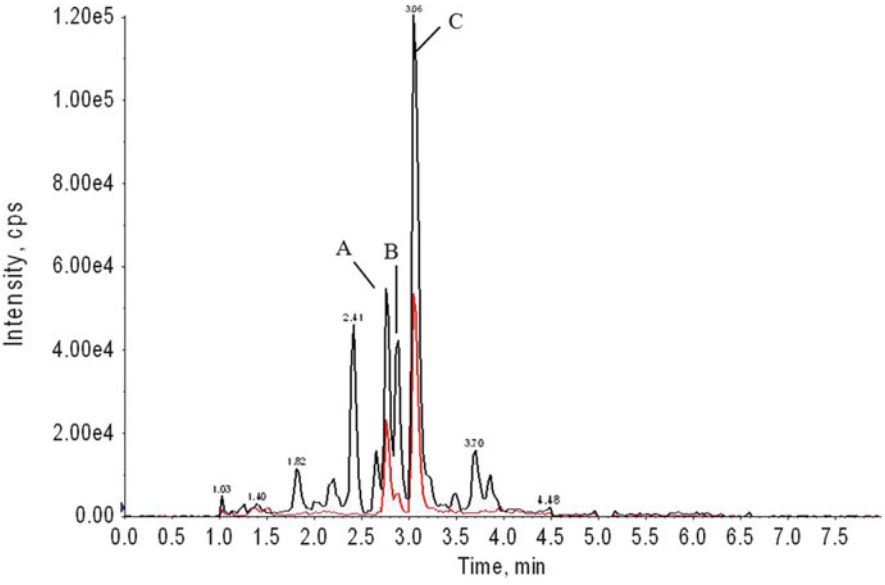

Fig. 8 Chromatogram of the quantifier (*black trace*; *m/z* 346 → 152) and the identifier (*red trace*; *m/z* 346 → 135) of cGMP in a murine bladder sample. Signals A, B, and C show the cGMP-specific quantifier/identifier ratio, with signal C occurring at the expected retention time. Thus signal C can be identified as 3′,5′-cGMP, whereas signal A can be assigned to 2′,3′-cGMP. The identity of signal B is still unresolved

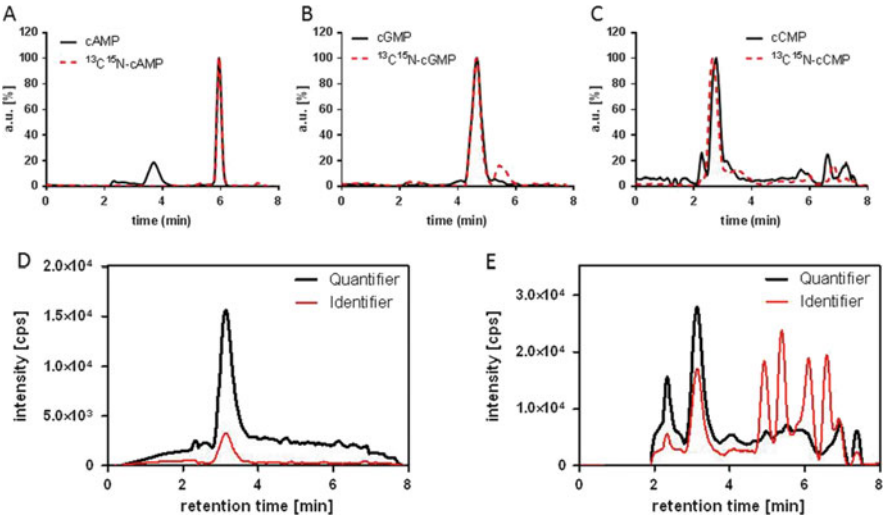

Fig. 9 Identification of cNMPs in human urine. (**a**) Quantifier mass transition of cAMP and $^{13}C^{15}$N-cAMP, (**b**) quantifier mass transition of cGMP and $^{13}C^{15}$N-cGMP, (**c**) quantifier mass transition of cCMP and $^{13}C^{15}$N-cCMP, (**d**) quantifier and identifier mass transition of cCMP in a standard sample, (**e**) quantifier and identifier mass transition of cCMP in a human urine sample

5.2 Identification of Cyclic Nucleotides in Human Urine

This analysis was carried out after a sample preparation procedure using the isotope-labeled internal standards $^{13}C_{10}{}^{15}N_5$-cAMP, $^{13}C_{10}{}^{15}N_5$-cGMP, and $^{13}C_9{}^{15}N_3$-cCMP. For cAMP and cGMP the peaks of the cNMP and the corresponding internal standard do overlap (Fig. 9) allowing the unequivocal identification of those cNMPs. On the other hand, the $^{13}C_9{}^{15}N_3$-cCMP- and the cCMP-signal show a difference in retention time of 0.1 min, which gives a first indication that this "cCMP"-signal does not belong to cCMP. In those cases, a comparison of the quantifier/identifier ratios is highly recommended. Figure 9 shows that for putative cCMP signal in the urine sample this ratio amounts to 1:4, whereas the ratio in a cCMP standards sample is 1:2. Both findings, the mismatch of the retention time and the differing quantifier/identifier ratios, suggest that cCMP is not present in human urine at detectable amounts.

6 Conclusions and Perspectives

The main recommendations regarding critical steps in cNMP analysis by HPLC-MS/MS are (1) adequate sample preparation steps resulting in an instant stop of cellular metabolism and extensive removal of interfering matrix components, (2) selection of robust and reproducible HPLC conditions, and (3) implementation

of reliable MS/MS recordings. However, due to the comparatively low mass accuracy of triple quadrupole mass spectrometers, high resolution but less sensitive mass spectrometers such as time-of-flight systems should be additionally applied in cNMP research (Bähre and Kaever 2014; Bähre et al. 2015; Dittmar et al. 2015). The described HPLC-MS/MS methods for cNMP can easily be upgraded with respect to further nucleoside metabolites.

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Medicinal Chemistry of the Noncanonical Cyclic Nucleotides cCMP and cUMP

Frank Schwede, Andreas Rentsch, and Hans-Gottfried Genieser

Contents

Abstract

After decades of intensive research on adenosine-3′,5′-cyclic monophosphate (cAMP)- and guanosine-3′,5′-cyclic monophosphate (cGMP)-related second messenger systems, also the noncanonical congeners cyclic cytidine-3′,5′-monophosphate (cCMP) and cyclic uridine-3′,5′-monophosphate (cUMP) gained more and more interest. Until the late 1980s, only a small number of cCMP and cUMP analogs with sometimes undefined purities had been described. Moreover, most of these compounds had been rather synthesized as precursors of antitumor and antiviral nucleoside-5′-monophosphates and hence had not been tested for any second messenger activity. Along with the recurring interest in cCMP- and cUMP-related signaling in the early 2000s, it became evident that well-characterized small molecule analogs with reliable purities would serve as highly valuable tools for the evaluation of a putative second messenger role of cyclic pyrimidine nucleotides. Meanwhile, for this purpose

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new cCMP and cUMP derivatives have been developed, and already known analogs have been resynthesized and highly purified. This chapter summarizes early medicinal chemistry work on cCMP and cUMP and analogs thereof, followed by a description of recent synthetic developments and an outlook on potential future directions.

Keywords
cCMP · cCMP analogs · cUMP · cUMP analogs · cCMP agarose · cUMP agarose · DB-cCMP · Prodrugs · Lipophilicity

1 Introduction

cAMP and cGMP are purine base-containing cyclic nucleotides and well-accepted second messengers (Fig. 1).

More than 1500 analogs of cAMP and cGMP have been synthesized during the last six decades since their discovery as endogenous molecules in 1957 and 1963, respectively (Sutherland and Rall 1957, 1958; Ashman et al. 1963), reflecting their importance as ubiquitous second messengers and as therapeutic targets. Chemical syntheses of the pyrimidine base-containing cyclic nucleotides cCMP and cUMP (Fig. 1) were reported in 1961 (Smith et al. 1961). Subsequently, cCMP was detected in the acid-soluble fraction and in hot water extracts of leukemia L-1210 cells (Bloch 1974). cUMP was isolated from rat liver extracts (Bloch 1975), and cUMP-degrading phosphodiesterase activities were found in dog heart (Hardman and Sutherland 1965) and in fat cells (Klotz and Stock 1971). In parallel, cCMP and

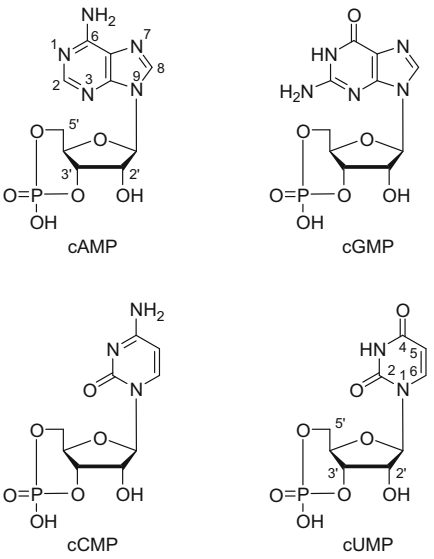
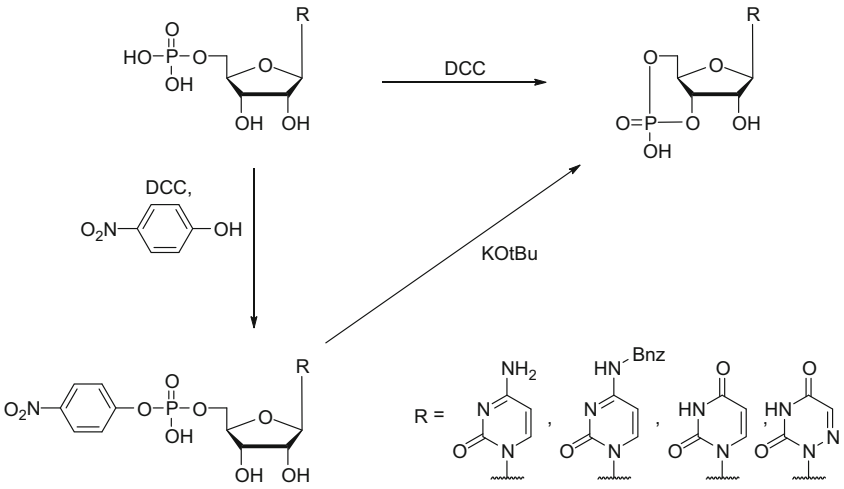


Fig. 1 Chemical structures of the canonical and noncanonical cyclic nucleotides cAMP, cGMP, cCMP, and cUMP. The structures depict the free acid form of the cyclic phosphate moiety. At physiological pH, the cyclic phosphate is negatively charged

cUMP were identified as minor cyclic nucleotide components besides cAMP, 2′-deoxy-cAMP, and cGMP in supernatants of *Corynebacterium murisepticum* and *Microbacterium* species (Ishiyama 1975). During the following years, cyclic pyrimidine nucleotides became not as popular as their purine cousins but suffered from setbacks probably due to scientific controversies about insensitive or imperfect analytical methods (compare Introduction chapter). Until the late 1980s, only a small set of cCMP and cUMP analogs had been synthesized, and the whole research field did not attract much attention until after the turn of the millennium.

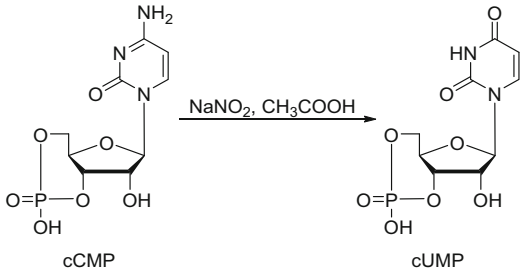
2 Early Syntheses of cCMP and cUMP

Multiple procedures for the synthesis of purine-based 3′,5′-cyclic monophosphates have also been employed for the synthesis of cCMP and cUMP. These strategies commonly involved direct cyclization of a nucleoside-5′-monophosphate precursor with carbodiimides, such as dicyclohexylcarbodiimide (DCC) (Scheme 1), isocyanates, or ynamines as dehydrating reagents, while applying high dilutions to reduce competing intermolecular coupling reactions (Smith et al. 1961; Naito and Sano 1965; Fujimoto and Naruse 1968). Alternatively, the 5′-monophosphate function was transformed into a 5′-(4-nitrophenyl)-active ester prior to potassium *tert*-butanolate (KOtBu)-initialized cyclization, leading to good yields of cCMP and cUMP (Scheme 1) (Borden and Smith 1966). These and additional general procedures were also applied to prepare the 2′-deoxy variants of cCMP and cUMP (Drummond et al. 1964). They are reviewed elsewhere in more detail (Simon et al. 1973; Miller and Robins 1976; Revankar and Robins 1982).



Scheme 1 Cyclization procedures employing cytidine- and uridine-5′-monophosphates to generate pyrimidine nucleoside-3′,5′-cyclic monophosphates

Scheme 2 Synthesis of cUMP by deamination of cCMP



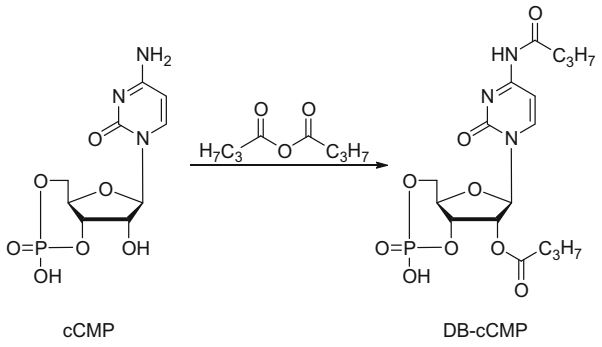
cUMP was also prepared by deamination of cCMP with sodium nitrite ($NaNO_2$) in aqueous acetic acid (Scheme 2) (Bloch 1975).

3 Early Analogs of cCMP and cUMP

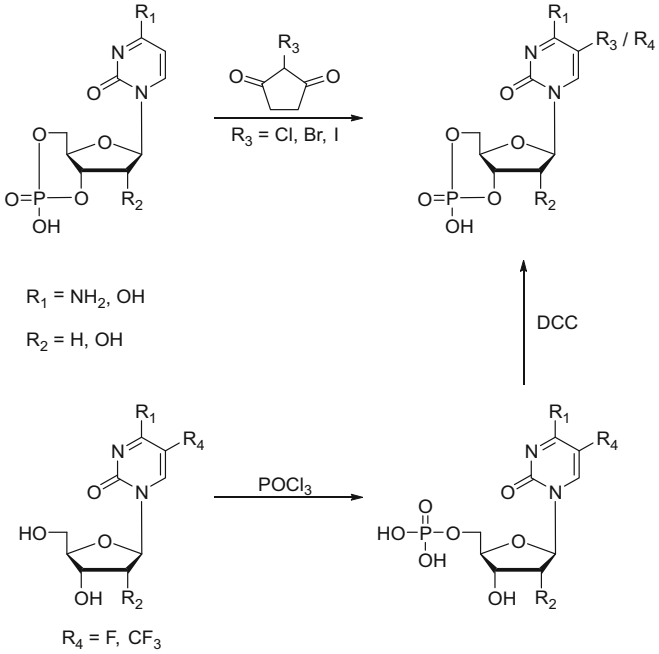
N^4-Benzoyl-cCMP (4-Bnz-cCMP) was the first analog of cCMP and synthesized from lipophilic N^4-benzoylcytidine-5′-monophosphate as an intermediate during cCMP synthesis (Smith et al. 1961) (Scheme 1). By contrast, unprotected cytidine-5′-monophosphate was only modestly soluble in pyridine/dimethylformamide (DMF), and the outcome of DCC-assisted cyclizations was low (Zielinski et al. 1974; Wierenga and Woltersom 1977). To the best of our knowledge, 4-Bnz-cCMP was never tested for biological activity but only obtained as a synthetic precursor for the synthesis of cCMP itself. N^4,2′-O-Dibutyrylcytidine-3′,5′-cyclic monophosphate (DB-cCMP) was prepared from cCMP with butyric acid anhydride in pyridine (Scheme 3) (Wierenga and Woltersom 1977). It became the first commercially available cCMP analog for biological testing, and hence its properties could be investigated in a number of studies (Yanagida et al. 1990; Ervens and Seifert 1991; Desch et al. 2010; Wolfertstetter et al. 2015). The same synthetic method was used to generate 2′-O-monobutyryluridine-3′,5′-cyclic monophosphate (2′-O-MB-cUMP) from cUMP (Posternak and Weimann 1974). DCC reaction with 6-azauridine-5′-monophosphate as starting material was reported to lead to 6-aza-cUMP in high yields (Holý et al. 1965) (Scheme 1).

In the 1960s and 1970s, pyrimidine bases modified in position 5 and corresponding nucleoside analogs like 5-fluorouracil and 5-fluoro-2′-deoxyuridine gained significant interest as antitumor and antiviral agents. As a follow-up, a number of 5-halo- and 5-alkyl-substituted cCMP and cUMP analogs were prepared and tested for their biological properties as chemotherapeutic compounds. Direct fluorination of cUMP with trifluoromethyl hypofluorite in cold methanolic solution followed by alkaline hydrolysis produced 5-F-cUMP in high yield (Robins et al. 1975). *N*-bromosuccinimide (NBS) treatment of cUMP in glacial acetic acid yielded 5-Br-cUMP as major product (Long and Robins 1978) (Scheme 4).

A similar strategy of direct halogenation was applied to prepare 5-Cl-cCMP, 5-Br-cCMP, 5-I-cCMP (Béres et al. 1985), 5-Cl-cUMP, 5-I-cUMP (Béres et al. 1986b), 5-Br-2′-deoxy-cUMP, and 5-I-2′-deoxy-cUMP (Béres et al. 1986c). In addition, 5-F-cCMP, 5-F-cUMP, 5-F-2′-deoxy-cUMP, and 5-CF_3-2′-deoxy-cUMP



Scheme 3 Synthesis of N^4,2′-O-dibutyrylcytidine-3′,5′-cyclic monophosphate (DB-cCMP) from cCMP



Scheme 4 Synthesis of position 5-halogenated cCMP and cUMP analogs and their 2′-deoxy congeners

were synthesized from corresponding cytidine and uridine analogs via selective 5′-phosphorylation with phosphoryl chloride and subsequent DCC cyclization of the *N*,*N*′-dicyclohexyl-4-morpholinecarboxamidine salts (Béres et al. 1985, 1986b, c) (Scheme 4). The latter method was also used to prepare several 5-alkyl-2′-deoxy-cUMP and 5-alkyl-cCMP analogs with methyl, ethyl, isopropyl, propyl, butyl, pentyl, hexyl, and octyl substituents (Béres et al. 1986a, 1989) (Fig. 2).

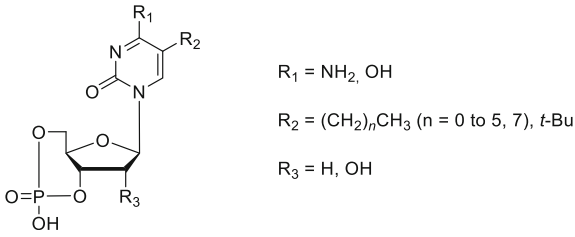


Fig. 2 Position 5-modified cCMP and cUMP analogs with alkyl substituents of various lengths

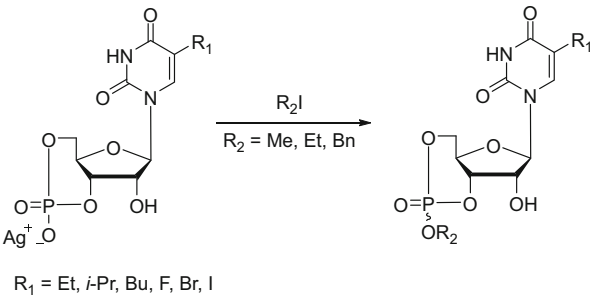
Albeit moderate antiviral and cytostatic activity was detected for some 5-substituted cUMP and 5-substituted 2′-deoxy-cUMP analogs, the corresponding uridine-based nucleosides or 5′-monophosphates showed 10-100 times higher potencies. In order to mask the negative charge of the cyclic phosphate and to further increase membrane permeability of 5-substituted 2′-deoxy-cUMP analogs, a series of neutral methyl, ethyl, and benzyl esters of the cyclic monophosphate was prepared. Syntheses were performed in DMF with the silver salt of 2′-deoxy-cUMP and excess alkyl or benzyl iodide to yield diastereomeric mixtures of axial and equatorial triesters (Scheme 5) (Béres et al. 1984).

Biological testing of these alkyl ester prodrugs revealed negligible antiviral and antitumor activities, indicating significant hydrolytic stability and biological inactivity of these aliphatic alkyl esters. The aromatic benzyl esters 5-I-2′-deoxy-cUMP-Bn and 5-iPr-2′-deoxy-cUMP-Bn showed ~10-fold higher activity but were still only as potent as their phosphate diester mother compounds at best. Additional cUMP alkyl and aralkyl triesters were prepared by direct treatment of the free acid form of cUMP and cUMP analogs with appropriate diazoalkanes (Scheme 6) (Engels and Pfleiderer 1975; Engels and Hoftiezer 1977).

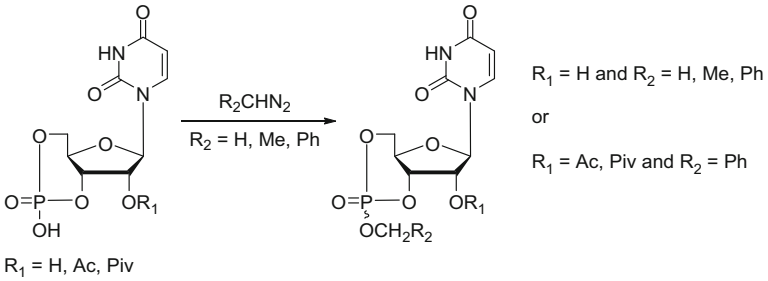
In summary, cCMP and cUMP analogs, including alkyl and benzyl ester prodrugs, were neither effective on their own nor able to serve as decent precursors of their biologically active 5′-monophosphate or nucleoside metabolites in multiple cell systems (Béres et al. 1986a, c). Given the laborious preparation procedures for many compounds of this type of prodrugs with dangerous reagents and lack of superior biological potency, research in this field had not been pursued further until very recently.

2′-O-Monosuccinylcytidine-3′,5′-cyclic monophosphate (2′-O-MS-cCMP) is formed with high selectivity upon treatment of cCMP with succinic acid anhydride in water and is used as reagent for cCMP radioimmunoassays (Scheme 7).

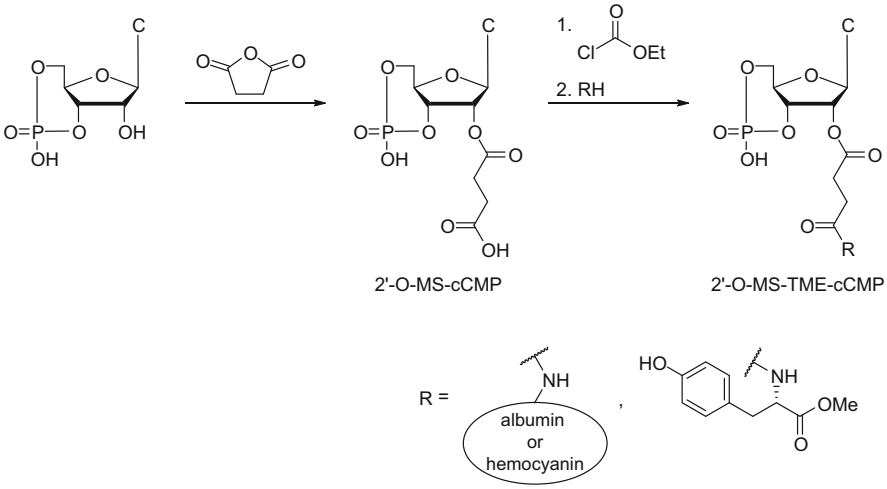
For antigen preparation, 2′-O-MS-cCMP was activated with ethyl chloroformate in DMF and coupled to serum albumin or hemocyanin. Chloroformate-activated 2′-O-MS-cCMP was also coupled to tyrosine methyl ester hydrochloride to yield 2′-O-MS-cCMP tyrosylmethyl ester (2′-O-MS-TME-cCMP) (Scheme 7), the starting material for subsequent radio iodination with $^{125}I^-$ and chloramine-T (Wierenga and Woltersom 1977; Cailla et al. 1978; Murphy and Stone 1979; Wikberg and Wingren 1981; Greenwood and Hunter 1963; Sato et al. 1982; Yamamoto et al. 1982).



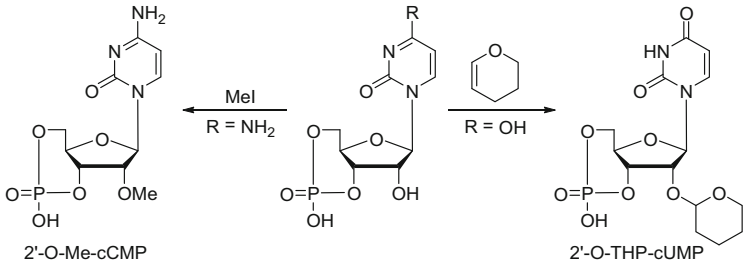
Scheme 5 Introduction of ester prodrug groups into 5-modified cUMP analogs



Scheme 6 Introduction of ester prodrug groups into cUMP analogs by diazoalkanes



Scheme 7 Syntheses of cCMP analogs appropriate for the development of immunological assays



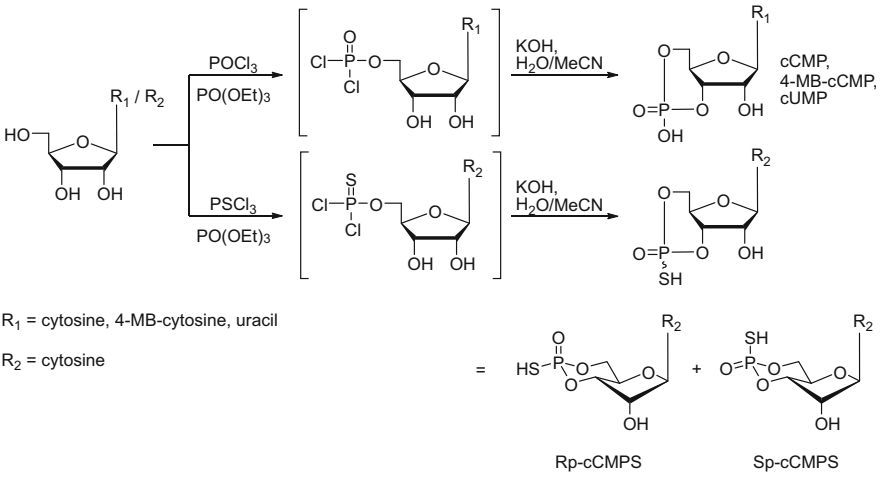
Scheme 8 Modification of cCMP and cUMP at 2′-position of the ribose

Other 2′-modified analogs of pyrimidine-based nucleoside-3′,5′-cyclic monophosphates (cNMPs) were described as well. 2′-O-Methyl-cCMP was synthesized with methyl iodide in DMF/water at alkaline pH (Tazawa et al. 1972), and 2′-tetrahydropyranyl-cUMP (2′-O-THP-cUMP) was generated upon reaction of cUMP with excess dihydropyran in anhydrous dioxane (Scheme 8) (Smith and Khorana 1959).

Cytosine- and uracil-based nucleoside-3′,5′-cyclic monophosphates with less common sugar moieties such as 1-(β-D-arabinofuranose) (Long et al. 1972), 1-(β-D-xylofuranose) (Holý and Sorm 1969), or 1-(β-D-lyxofuranose) (Ukita and Hayatsu 1961) have been described and are excellently reviewed by Revankar and Robins (Revankar and Robins 1982). 2′,3′-Seco-3′,5′-cCMP and 2′,3′-seco-3′,5′-cUMP with ring-opened ribose units were prepared via the corresponding 5′-monophosphates with DCC-assisted cyclization in pyridine. Treatment of 2′,3′-secocytidine with phosphoryl chloride in triethylphosphate was reported as alternative synthetic route (Lassota et al. 1986).

4 Subsequent Cyclization Methods for cCMP and cUMP and Analogs

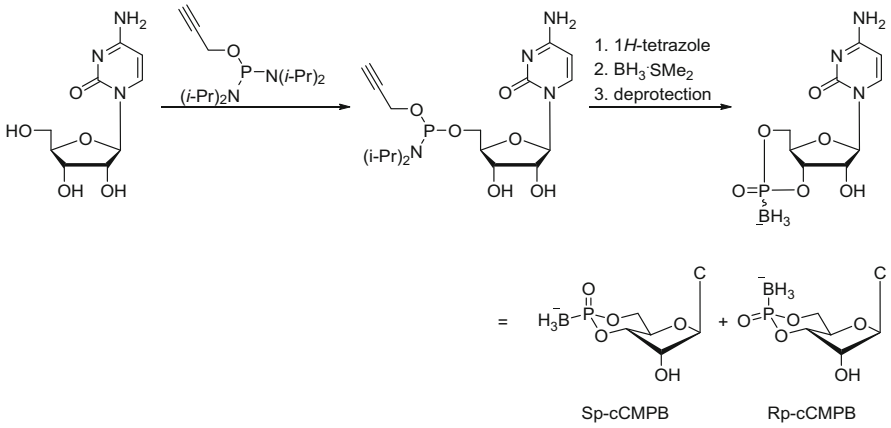
A rather convenient access to cyclic monophosphates consisting of a one-pot two-step 3′,5′-cyclization synthesis with unprotected purine nucleosides as starting material was developed by one of us (Genieser et al. 1989). This method employed cheap nucleosides without the need for laborious purification of 5′-phosphorylated intermediates prior to cyclic phosphate formation, resulting in a fast and straightforward alternative to the classical methods of Smith and Borden (Smith et al. 1961; Borden and Smith 1966). As a follow-up project, in the late 1990s, our lab became interested in transferring this synthesis to pyrimidine-based cyclic nucleotides. Based on the pioneering phosphorylation studies of Yoshikawa and coworkers, unprotected cytidine and uridine, respectively, were regioselectively activated by phosphoryl chloride in 5′-position of the ribose in triethylphosphate under anhydrous conditions (Scheme 9) (Kusashio and Yoshikawa 1968; Yoshikawa et al. 1969).



Scheme 9 Regioselective phosphorylation/thiophosphorylation of pyrimidine nucleosides and subsequent cyclization to 3′,5′-cyclic monophosphates and diastereomeric monophosphorothioates. For better comparison, the sterically distinct equatorial (Rp-cCMPS) and axial (Sp-cCMPS) isomers are also depicted in perspectival drawing

The presumably formed 5′-dichloridate intermediate was cyclized in situ under alkaline conditions by dropwise addition into a large volume of vigorously stirred alkaline aqueous acetonitrile. With this procedure it was possible to generate cCMP and cUMP in ~30% isolated yields in multi-gram scale with excellent purities of > 99% (HPLC) (Scheme 9) (Schwede and Genieser, unpublished results). Earlier commercial preparations repeatedly suffered from insufficient purities and typically had to be repurified (Bloch 1974, 1975). The ameliorated availability of larger amounts of pyrimidine-based cyclic nucleotides coincided well with the revival of cCMP- and cUMP-related second messenger research, mainly driven by the labs of Russell Newton in Swansea, UK (Newton 1995; Newton et al. 1997, 1999), and Roland Seifert in Hannover, Germany (Ervens and Seifert 1991; Desch et al. 2010; Hartwig et al. 2014).

A similar synthetic approach, albeit with thiophosphoryl chloride in the first step, was used to prepare analogs with sulfur modifications at the cyclic phosphate moiety (Scheme 9). Originally, this thiocyclization method was used to prepare the diastereomeric mixture of adenosine-3′,5′-cyclic monophosphorothioate (cAMPS) (Genieser et al. 1988). Rp-cAMPS, with a sulfur atom replacing oxygen in the equatorial exocyclic position of the 3′,5′-cyclic monophosphate, was the first antagonist of cAMP-dependent protein kinase (PKA). Sp-cAMPS, the corresponding diastereomer with the sulfur in axial position, was shown to be an activator of PKA (O'Brian et al. 1982; de Wit et al. 1984). Also, phosphorothioate versions of cGMP were prepared (Rp-/Sp-cGMPS) which show similar biological effects toward cGMP-dependent protein kinase (PKG) (Schwede et al. 2000b). The terms "Rp" and "Sp" determine the different isomers of the chiral phosphorus atom

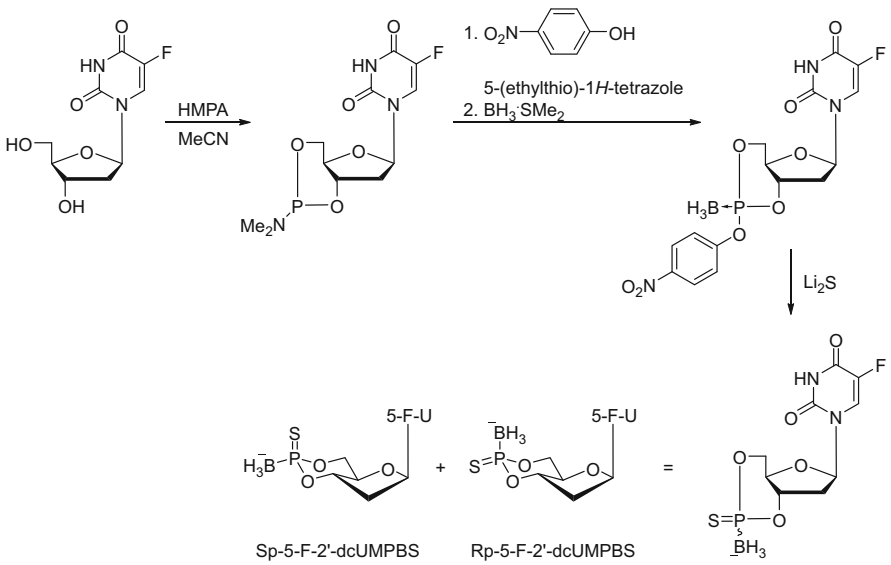


Scheme 10 Synthesis of diastereomeric cCMP analogs with boranophosphate modification

in the 3′,5′-cyclic monophosphorothioate according to the Cahn-Ingold-Prelog priority rules (CIP). Rp and Sp isomers of cAMPS and cGMPS analogs have significantly improved stability against all mammalian phosphodiesterase families tested so far (Poppe et al. 2008), an important practical advantage of activatory or inhibitory pharmacological tools for the study of cAMP and cGMP signaling pathways. The two-step thiocyclization procedure was adapted to pyrimidine nucleosides (Scheme 9), and Rp-cCMPS and Sp-cCMPS were prepared in isolated yields of ~15% and 10%, respectively. Meanwhile, both isomers are commercially available for biological testing. These cCMPS analogs, their future lipophilic congeners, and the corresponding cUMPS analogs are expected to have enhanced PDE stability and thus should be especially suitable for long-term incubations in cell culture or tissue experiments.

Within a series of boranophosphate-modified cyclic nucleotide analogs (Genieser 2012), also the isomers of cytidine-3′,5′-cyclic monoboranophosphate (cCMPB) have been prepared (Scheme 10).

In a cyclic boranophosphate, one of the exocyclic oxygen atoms is replaced by a borano group (BH_3), and hence axial and equatorial isomers must be distinguished. However, the CIP rules here lead to interchanged R and S designations compared to phosphorothioates. Following a published protocol (Lin et al. 2001), the 5′-position of cytidine was selectively phosphitylated by 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite in dry DMF, cyclized by 1*H*-tetrazole without isolation of the intermediate, and boronated with borane-dimethyl sulfide complex. Rp- and Sp-cCMPB were isolated in rather moderate yields of 20% and 18% (Scheme 10). Both, the axially boronated isomer (Rp-cCMPB) and its diastereomeric counterpart with an equatorial borano group (Sp-cCMPB), turned out to be considerably more lipophilic compared to the corresponding phosphorothioates (Rp- and Sp-cCMPS) but still only reach a level comparable to that of cAMP. Besides its considerable impact on lipophilicity, the boranophosphate modification



Scheme 11 Synthesis of Rp- and Sp-5-fluoro-2′-deoxyuridine-3′,5′-cyclic monoboranophosphorothioate

leads to high stability toward both oxidative degradation and hydrolysis by phosphodiesterases (Genieser 2012). The biological effects of both analogs have not been explored yet, but by deduction it can be expected that they behave like Rp- and Sp-cCMPS with even improved metabolic stability. Thus, the boranophosphate modification could be of interest, if the second messenger potential of cCMP shall be used and a stable and long-lasting signal is required. However, the still insufficient lipophilicity would require either additional prodrug strategies or other transport vehicles and techniques for intracellular uptake. Synthesis of the related 5-fluoro-2′-deoxyuridine-3′,5′-cyclic monoboranophosphorothioate (cFdUMPBS, 5-F-2′-dcUMPBS) was reported by Li and Ramsay Shaw (Scheme 11) (Li and Shaw 2002).

The authors explored that direct cyclization attempts of preformed boranophosphorothioates in 3′- or 5′-position of the ribose were unsuccessful. To overcome this obstacle, a one-pot synthesis with 5-fluoro-2′-deoxyuridine-3′,5′-cyclic monophosphorodimethylamidite as key intermediate was developed. Under strictly anhydrous conditions, 5-fluoro-2′-deoxyuridine dissolved in acetonitrile was reacted with hexamethylphosphoramide (HMPA) to form the reactive 3′,5′-cyclic phosphoramidite. This intermediate was transformed to the 4-nitrophenyl phosphate triester with 4-nitrophenol and 5-ethylthio-1*H*-tetrazole, followed by subsequent boronation with borane-dimethyl sulfide complex. After final thiation with lithium sulfide in DMF/dioxane, 5-F-2′-dUMPBS was prepared in 65–70% overall yield as a mixture of Rp and Sp diastereomers (Scheme 11) (Li and Shaw 2002).

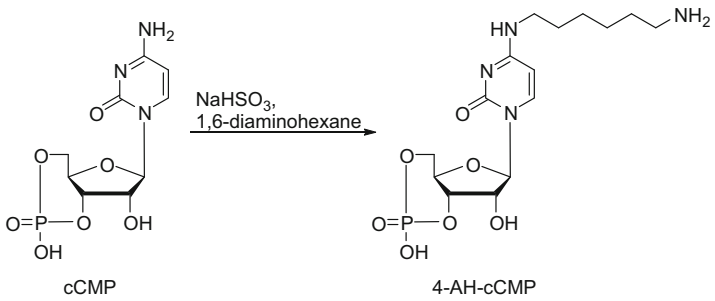
5 Subsequent Syntheses of cCMP and cUMP Analogs

During the last few years, a major task in the field was to solve the question as to whether or not cCMP and cUMP act as second messenger molecules in the cell. This implies not only their endogenous formation, which is reviewed in Chapter Mass-spectrometric analysis of non-canonical cyclic nucleotides of this book series, but also the isolation and identification of receptor proteins of cCMP and cUMP in order to confirm any second messenger properties of these pyrimidine cyclic nucleotides. The chemical proteomics approach with cAMP and cGMP agaroses as affinity baits has been proven useful either to purify overexpressed PKA regulatory subunits (Bertinetti et al. 2009), to confirm known primary receptors of cAMP/cGMP or to identify new PKA- or PKG-interacting secondary proteins (Hanke et al. 2011; Kim and Park 2003; Scholten et al. 2006). Therefore, we were aiming to design a set of cCMP and cUMP analogs bearing functionalized spacers in different positions for subsequent coupling to agaroses. In cCMP, the N^4 position and the 2′-OH position were selected to introduce 6-aminohexyl spacers, with a primary terminal amino group (ω-amino group) to enable further chemical transformations. In cUMP, an aminoallyl group for position C-5 and a 6-aminohexyl unit for 2′-OH were used as functional spacers. N^4-(6-Aminohexyl) cytidine-3′,5′-cyclic monophosphate (4-AH-cCMP) was prepared from cCMP by a bisulfite-catalyzed transamination with 1,6-diaminohexane in aqueous solution at neutral pH (Scheme 12) that is selective for the cytosine base compared to adenine, guanine, thymine, or uracil, according to a literature procedure for cytidine-2′(3′)-monophosphate (Scofield et al. 1977).

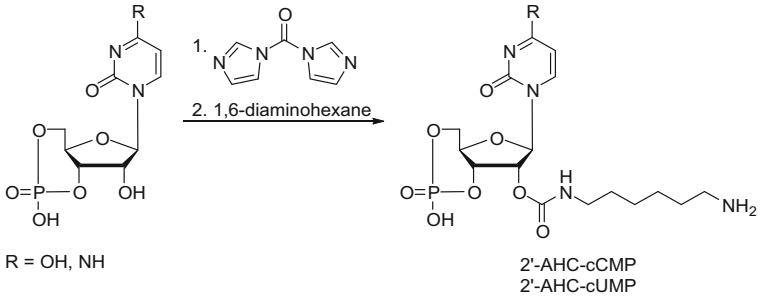
2′-O-(6-Aminohexylcarbamoyl)cytidine-3′,5′-cyclic monophosphate (2′-AHC-cCMP) and 2′-O-(6-aminohexylcarbamoyl)uridine-3′,5′-cyclic monophosphate (2′-AHC-cUMP) were synthesized from cCMP and cUMP in analogy to Corrie et al. employing initial activation with 1,1′-carbonyldiimidazole in anhydrous DMF, followed by addition of excess 1,6-diaminohexane (Scheme 13) (Corrie et al. 1992).

Commercially available 5-trifluoracetyl-(3-aminoallyl)uridine was the starting material to prepare 5-(3-aminoallyl)uridine-3′,5′-cyclic monophosphate (5-AA-cUMP) via selective 5′-phosphorylation in triethylphosphate and cyclization in aqueous acetonitrile as described above (compare Scheme 9). In the final step of the procedure, the trifluoracetyl protection group was removed under alkaline conditions (Scheme 14) (Genieser et al. 1989).

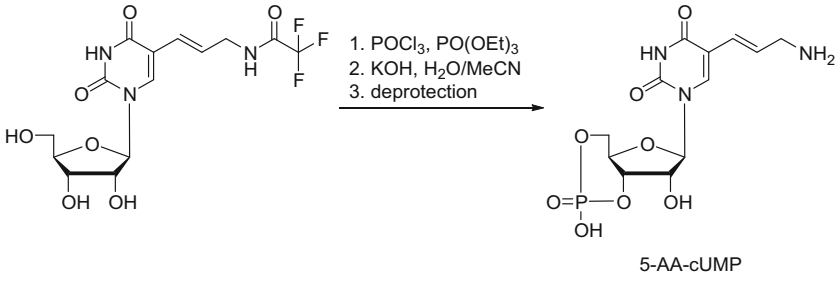
The terminal aliphatic amino function in these pyrimidine base cyclic nucleotides can serve as the reactive center to enable coupling reactions with activated agaroses or further derivatization with reporter groups such as fluorescent dyes, spin labels, or biotin and digoxigenin labels. For agarose coupling, the reactive amine-containing cyclic nucleotides were incubated with *N*-hydroxysuccinimide (NHS) ester-activated agarose beads in anhydrous DMSO and a non-nucleophilic base like diisopropylethylamine according to a literature procedure (Fig. 3) (Bertinetti et al. 2009).



Scheme 12 Introduction of a hexyl spacer with a reactive ω-amino group into N^4 position of cCMP



Scheme 13 Modification of cCMP/cUMP in the 2′-ribose position with a reactive hexyl spacer group



Scheme 14 Modification of cUMP with a reactive spacer group in base position 5

As described in Chapter cCMP and cUMP across the tree of life: From cCMP and cUMP generators to cCMP- and cUMP-regulated cell functions in this book series in more detail, 4-AH-cCMP agarose and 2′-AHC-cCMP agarose were suitable tools to bind and pull down PKA regulatory subunits in a number of cell lines (Hammerschmidt et al. 2012). Very recently, 4-AH-cCMP agarose beads were used to identify multiple cCMP binders in lung and jejunum tissue lysates from mice, including PKG, PKA, and mitogen-activated protein kinase (MAPK)

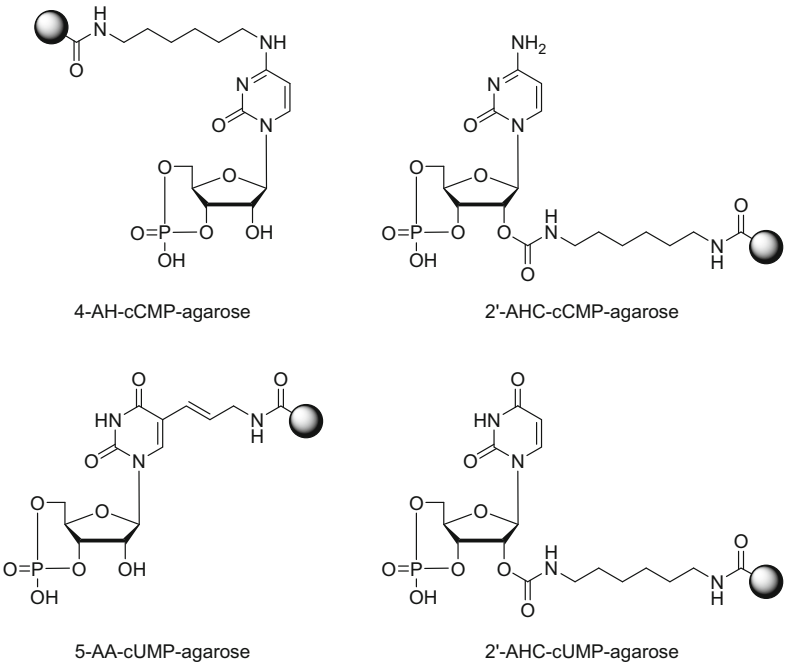
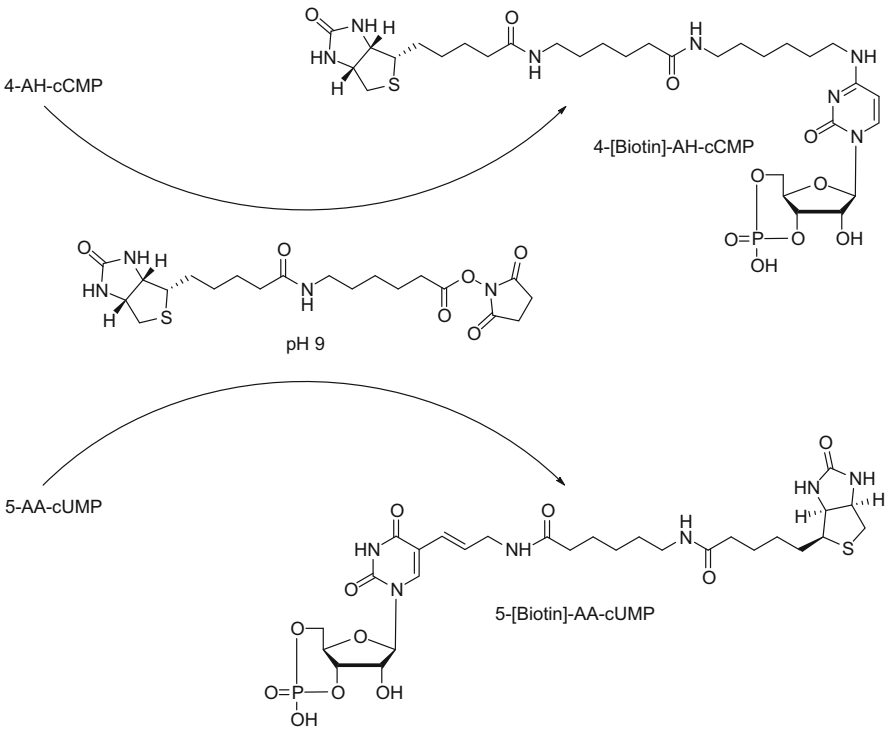


Fig. 3 Examples for commercially available affinity gels with cCMP and cUMP ligands

(Wolfertstetter et al. 2015) (see also Chapter cAMP-dependent protein kinase and cGMP-dependent protein kinase as cyclic).

A related aqueous NHS chemistry in sodium bicarbonate buffer at pH 9 was performed with biotinamidocaproate-NHS ester ([Biotin]-NHS) to prepare N^4-(6-[biotinyl]aminohexyl)cytidine-3′,5′-cyclic monophosphate (4-[Biotin]-AH-cCMP) and 5-(3-[biotinyl]aminoallyl)uridine-3′,5′-cyclic monophosphate (5-[Biotin]-AA-cUMP) as additional tools to study protein-ligand interactions and to identify putative receptor proteins (Scheme 15).

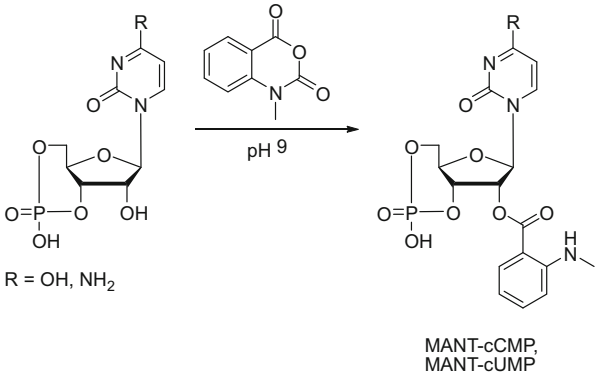
A biologically regulated signal inactivation mechanism is one in a number of other prerequisites for a second messenger. In case of nucleoside-3′,5′-cyclic monophosphates, corresponding 3′,5′-cyclic phosphodiesterases (PDEs) are responsible for signal termination by hydrolysis of cNMPs to nucleoside-5′-monophosphates. Since this is the only known metabolic pathway to control intracellular levels of cAMP and cGMP, PDEs have a major impact on the kinetics and termination of their second messenger signaling. The PDE superfamily consists of 11 classes with more than 100 isozymes described so far with different substrate profiles for cAMP/cGMP (Bender and Beavo 2006). Fluorescently labeled substrates are known tools for the investigation of enzymatic reactions and have been employed for the study of a number of PDEs (Hiratsuka 1982; Huang et al. 2002). Especially cAMP/cGMP analogs with fluorescent modifications in 2′-OH position became popular for the examination of PDEs, as this position is



Scheme 15 Modification of ω-amino-functionalized cCMP and cUMP analogs with biotin

usually not critical for substrate recognition and hydrolysis by the catalytic site (Bender and Beavo 2006; Holz et al. 2008). The *N*-methylanthranilic (MANT) dye is sensitive to its chemical environment, and 2′-O-(*N*′-methylanthraniloyl)adenosine-3′,5′-cyclic monophosphate (MANT-cAMP) and MANT-cGMP undergo a significant change in relative fluorescence upon hydrolytic cleavage of the cyclic monophosphate. Therefore, both compounds were valuable probes for the development of continuous PDE assays with fluorescence readout (Hiratsuka 1982; Johnson et al. 1987). In the emerging field of cCMP and cUMP research, corresponding MANT-cCMP/MANT-cUMP analogs were thought to be versatile probes to examine the hydrolysis of these pyrimidine cyclic monophosphates by multiple PDEs and to evaluate their role as putative second messengers. cCMP and cUMP were reacted with *N*-methylisatoic acid anhydride in aqueous solution at pH 9 to generate MANT-cCMP and MANT-cUMP (Scheme 16). This procedure led to raw mixtures with several fluorescent impurities, which were removed by repeated reversed-phase HPLC purification steps to avoid interferences in fluorescence assays. MANT-cUMP was successfully employed in endpoint assays and in continuous fluorescence PDE assays and found to be degraded by PDE3A, while MANT-cCMP was not hydrolyzed by any of the initially tested PDEs (PDE1, PDE3, PDE5, PDE9 isoforms) (Reinecke et al. 2013).

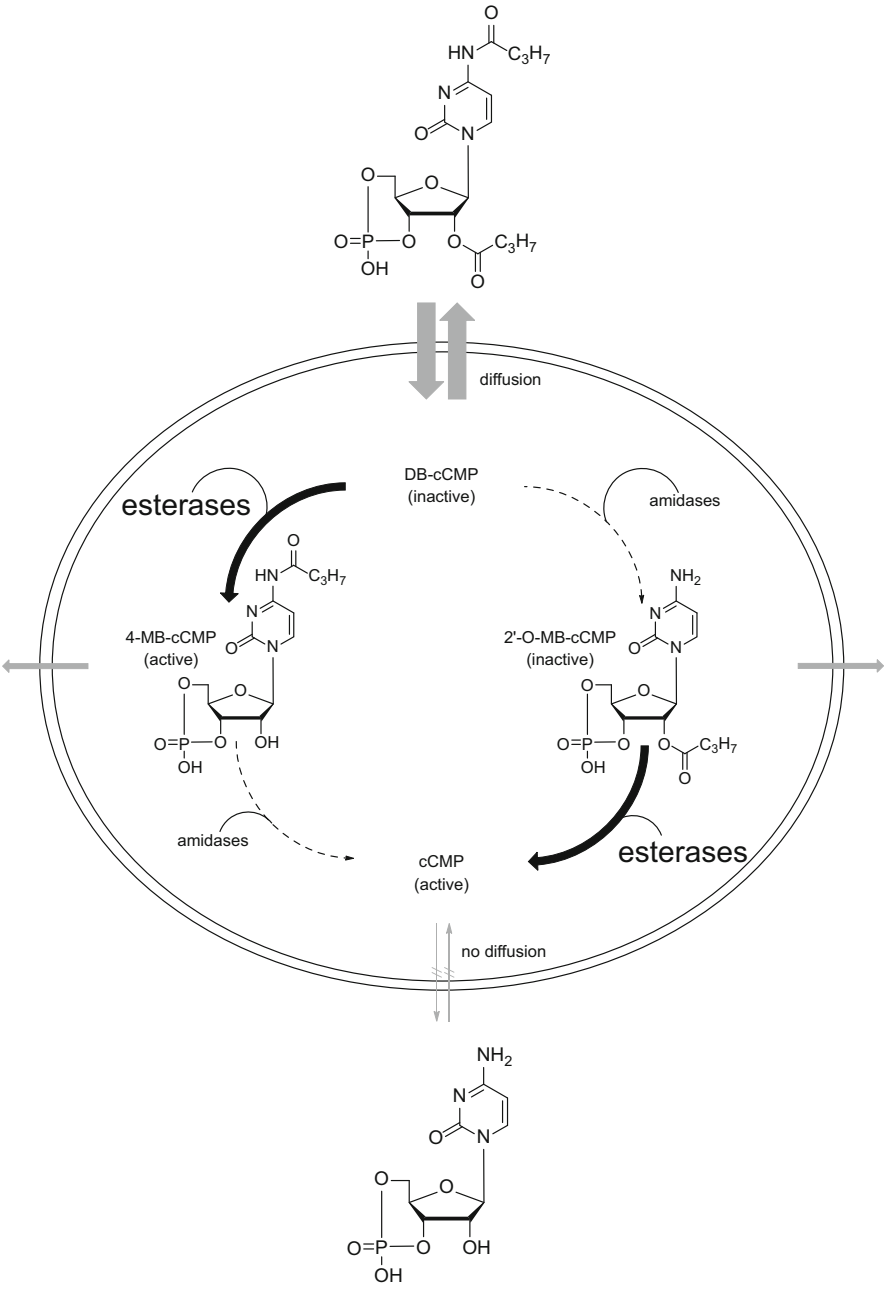
Scheme 16 Modification of the ribose 2′-position of cCMP/cUMP with the fluorescent MANT group



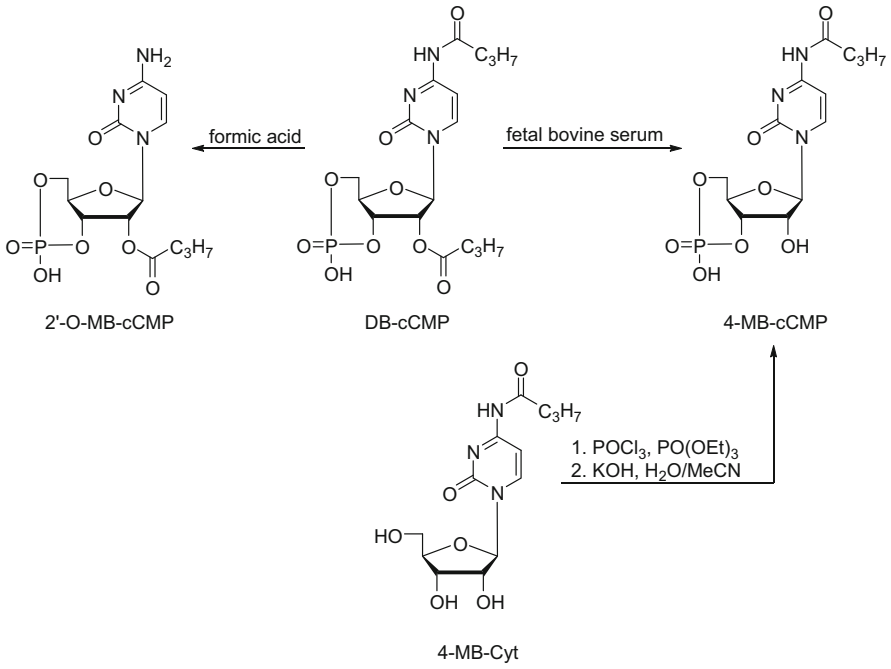
6 Subsequent Syntheses of cCMP and cUMP Prodrug Analogs

N^4,2′-O-Dibutyrylcytidine-3′,5′-cyclic monophosphate (DB-cCMP) (Scheme 3), one of the early cCMP analogs described in the 1970s (Wierenga and Woltersom 1977), can be recognized as a potential prodrug with reasonable lipophilicity and moderate membrane permeability allowing extracellular applications to cell cultures or tissues. Once inside the cell, DB-cCMP is thought to be a substrate of endogenous esterases that cleave off the 2′-butyrate, leading to N^4-monobutyryl-cytidine-3′,5′-cyclic monophosphate (4-MB-cCMP) with a free 2′-OH-group (Scheme 17). The liberated butyrate was described as a bioactive compound (Soldatenkov et al. 1998; Crane 2000), and reagents, such as sodium butyrate, are recommended probes for control experiments. The metabolic esterase step goes along with an increased polarity of 4-MB-cCMP and thus results in an impaired ability of 4-MB-cCMP to efficiently leave the cell by passive diffusion. As a consequence, a certain accumulation of 4-MB-cCMP can be achieved in the cytosol.

With the exception of PDEs and exchange proteins directly activated by cAMP (Epac), most receptor proteins of the second messengers cAMP and cGMP have a highly conserved glutamate in their cyclic nucleotide-binding domains for high-affinity binding. This glutamate forms an important hydrogen bond with the free ribose 2′-OH-group of cAMP and cGMP and their analogs (Schwede et al. 2000b; Taylor et al. 2008). By analogy, 4-MB-cCMP was speculated to serve as biologically active metabolite of DB-cCMP and to be a cross-activating activator of cGMP kinase I in the mouse aorta relaxation model and in platelets (Desch et al. 2010). However, it cannot be ruled out that 2′-O-monobutyrylcytidine-3′,5′-cyclic monophosphate (2′-O-MB-cCMP) and cCMP may be formed as alternative metabolites upon N^4-debutyrylation of DB-cCMP and 4-MB-cCMP by endogenous amidases. For further testing at isolated PKA and PKG isozymes and in cell culture, the potential metabolites of DB-cCMP, 4-MB-cCMP, and 2′-O-MB-cCMP were



Scheme 17 Schematic representation of a cell. Uptake of DB-cCMP by passive diffusion, followed by intracellular release of active metabolites 4-MB-cCMP and cCMP via enzymatic degradation steps. Arrow widths reflect distinct potencies to penetrate biological membranes with DB-cCMP $>>$ 4-MB-cCMP ~ 2′-O-MB-cCMP $>>$ cCMP as rank order from highest to lowest efficacy (compare also Table 1)



Scheme 18 Preparation of potential metabolites of DB-cCMP

prepared in our laboratory. DB-cCMP was reacted with 33% formic acid at elevated temperature to hydrolyze the acid-labile butyrate in N^4 position leading to the formation of 2′-O-MB-cCMP (Scheme 18). 4-MB-cCMP was initially prepared in small scale from DB-cCMP by chemoenzymatic 2′-O-debutyrylation with fetal bovine serum (Wolter et al. 2014). For large-scale production of 4-MB-cCMP in multi-gram amounts, N^4-monobutyrylcytidine (4-MB-Cyt) was 5′-phosphorylated and 3′,5′-cyclized according to Genieser and coworkers (Scheme 18) (Genieser et al. 1989).

In in vitro activation assays, only 4-MB-cCMP was able to activate PKA and PKG at micromolar concentrations comparable to cCMP, while DB-cCMP and 2′-O-MB-cCMP were not active even at millimolar doses. These results highlight 4-MB-cCMP as active metabolite of DB-cCMP in in vivo experiments (Wolter et al. 2011, 2014), although only HPLC-MS/MS analytics of the cytosolic content of a cell culture or tissue following incubations with DB-cCMP can provide a final proof. In summary, DB-cCMP fulfills the characteristics of a prodrug with no biological activity on its own and the need for enzymatic bioactivation, probably to 4-MB-cCMP. Even though DB-cCMP has improved hydrophobicity compared to cCMP, biological applications are restricted by its still modest membrane permeability. The negative charge of the cyclic phosphate moiety is a major cause of limited membrane permeability, a general caveat for the in vivo use of phosphate-containing small molecules.

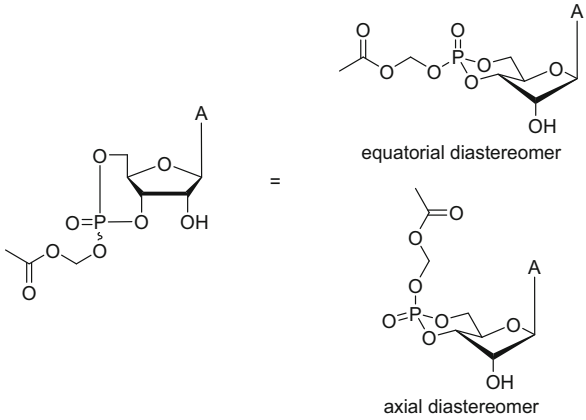
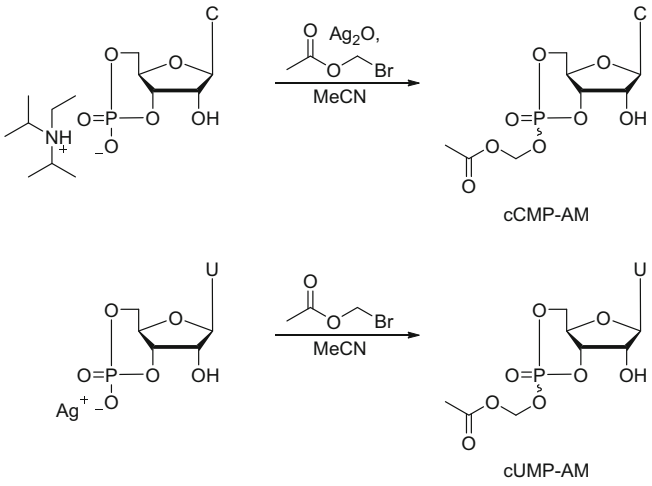


Fig. 4 Diastereomers of the prodrug cAMP-AM. The sterically distinct equatorial and axial isomers are also depicted in perspectival drawing

In the 1990s, the acetoxymethyl ester-protecting group was introduced into cyclic nucleotides by Schultz and coworkers, leading to uncharged and highly membrane-permeant cyclic phosphate triesters of cAMP and cGMP (Schultz et al. 1993; Schwede et al. 2000a; Schultz 2003). cAMP-acetoxymethyl ester (cAMP-AM) (Fig. 4) and cGMP-acetoxymethyl ester (cGMP-AM) and analogs thereof were shown to easily penetrate cellular membranes.

Once inside the cell, the AM-ester group is removed by endogenous esterases to generate the cyclic nucleotide mother compound, acetic acid, and formaldehyde. Phosphate tris(acetoxymethyl)ester (PO_4-AM_3) with three AM-ester groups connected to inorganic phosphate is a control reagent to evaluate potential biological effects of the released by-products acetic acid and formaldehyde (Schultz et al. 1993). After liberation of the negatively charged phosphate, the highly polar cyclic nucleotide is only hardly able to escape from the cell by passive diffusion and thus accumulates by a factor of 5 to 10 in the cell (Bartsch et al. 2003; Börner et al. 2011). Esterase bioactivation is highly efficient and fast, leading to 100-1000-fold improved biological activities of the AM-ester prodrugs within minutes (Vliem et al. 2008; Chepurny et al. 2009). Compared to the former cyclic nucleotide triesters containing aliphatic alkyl or aralkyl groups (Béres et al. 1984; Engels and Pfleiderer 1975) with only moderate biological activities, this new generation of cNMP-AM esters appeared to be highly potent and useful to study the in vivo second messenger signaling of cAMP and cGMP in multiple biological models.

As a logical consequence, we set out to transfer the underlying AM-ester chemistry to cCMP and cUMP to provide new analogs for biological testing of these putative second messengers. Under strictly anhydrous conditions, carefully dried cCMP, diisopropylethylammonium salt, was reacted with acetoxymethyl bromide and solid silver-I-oxide (Ag_2O) in absolute acetonitrile to produce cCMP-acetoxymethyl ester (cCMP-AM) in reasonable yields (>35%) as mixture of axial and equatorial isomers after isolation by semipreparative HPLC (Scheme 19) (Beckert et al. 2014).



Scheme 19 Synthesis of isomeric mixtures of acetoxymethyl ester-modified cCMP and cUMP

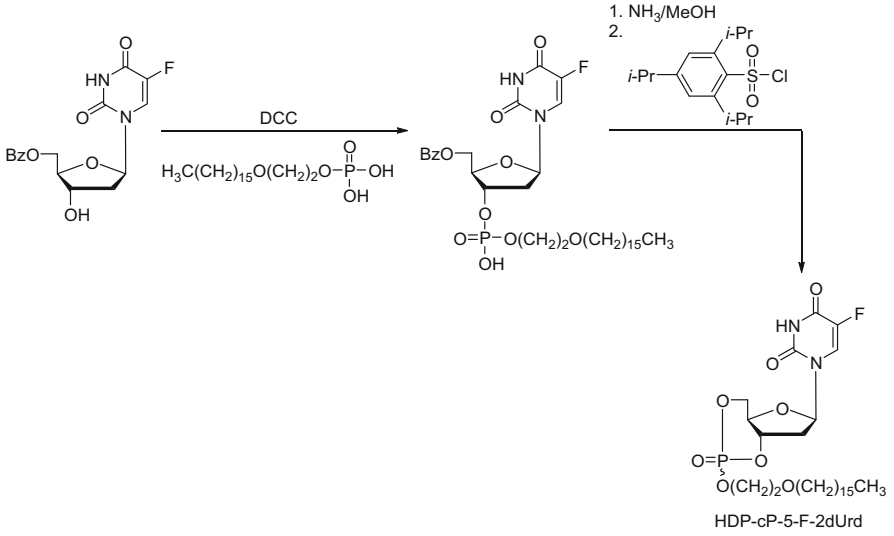
It turned out that the use of Ag_2O was imperative for a successful setup with concomitant formation of AgBr as driving force to increase synthetic output. Other published reaction conditions for cAMP-AM or cGMP-AM omitting Ag_2O led to significantly reduced yields below 10% and complex reaction mixtures (Schwede, unpublished results). In case of cUMP, its silver salt was treated with acetoxymethyl bromide in absolute acetonitrile to generate cUMP-acetoxymethyl ester (cUMP-AM) in isolated yields of $\leq 10\%$ (Scheme 19). Interestingly, addition of Ag_2O did not increase reaction yields but induced massive and irreversible product absorption on the solid silver matrix. cCMP-AM and cUMP-AM were subsequently used as in vivo tools to study and dissect the effects of bacterial nucleotidyl cyclase toxins ExoY from *Pseudomonas aeruginosa* and CyaA from *Bordetella pertussis* which produce cUMP and cCMP (Beckert et al. 2014) as described in detail in Chapter CyaA and edema factor as cNMP generators: Comparison with ExoY of this volume. cUMP-AM generated high intracellular levels of cUMP already after a period of 5 min in B103 neuroblastoma cells, as was verified by sophisticated HPLC-MS/MS quantification of cNMP levels (Beckert et al. 2014).

In parallel to the development of the first AM esters of cyclic nucleotides designed for a fast release of the bioactive mother compound, an alternative prodrug approach with a sustained release of pharmacophore has been established and brought to clinical testing by the group of Karl Hostetler. These compounds consist of tailor-made lipid esters of antiviral nucleoside monophosphonates, especially hexadecyloxypropyl-cidofovir (HDP-CDV, CMX-001, ongoing clinical phase III) and hexadecyloxypropyl tenofovir (HDP-tenofovir, CMX-157, completed clinical phase I). Due to their amphiphilic structure with a hydrophobic lipid part and a negative charge at the phosphonate, CMX-001 and CMX-157 display reasonable solubility in aqueous solution combined with excellent

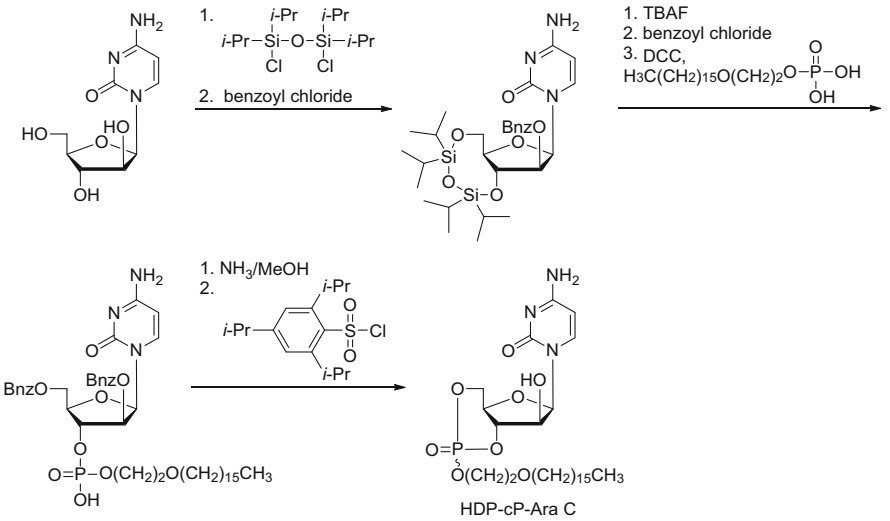
bioavailability probably through phospholipid uptake mechanisms in the small intestine (Painter and Hostetler 2004; Hostetler 2010). Proposed metabolic activation of these lipid esters is via phospholipase C or successive β-oxidation in peroxisomes (Hostetler 2009; Yoshida et al. 1990). Recently, this lipid ester concept was further adapted to 3′,5′-cyclic monophosphates of 5-fluoro-2′-deoxyuridine and cytarabine (Schemes 20 and 21), two established antiproliferative agents in use for the treatment of ophthalmologic diseases induced by pathological intraocular angiogenesis. The underlying strategy combines the principal HDP-ester prodrug with the cyclic nucleotide as the second-phase prodrug for the finally released 5′-monophosphate metabolite. The uncharged cyclic nucleotide ester HDP prodrugs are highly lipophilic and only barely soluble in aqueous media, rendering them to promising depot formulations for extremely slow release and prolonged drug exposure after intravitreal injection. Hexadecyloxypropyl 5-fluoro-2′-deoxyuridine-3′,5′-cyclic monophosphate (HDP-cP-5-F-2dUrd, 5-F-2′-d-cUMP-HDP) was prepared in four steps from 5-fluoro-2′-deoxyuridine via regioselective 5′-benzoylation, DCC-assisted coupling of hexadecyloxypropyl phosphate to the 3′-OH position, alkaline 5′-deprotection, followed by cyclization with 2,4,6-triisopropylbenzenesulfonyl chloride, and 1-methylimidazole in pyridine (Scheme 20) (Cheng et al. 2010).

After intravitreal injection of HDP-cP-5-F-2dUrd, the crystallized drug depot was visible for more than 14 weeks in the vitreous body, produced no detectable toxicities, and provided efficient protection against trauma-induced proliferative vitreoretinopathy (PVR) in rabbit (Cheng et al. 2010). The hexadecyloxypropyl prodrug of cytarabine-3′,5′-cyclic monophosphate (HDP-cP-AraC; ara-cCMP-HDP) was generated in a five-step procedure from cytarabine with standard protection group chemistry, DCC-assisted hexadecyloxypropyl phosphate coupling, and cyclization with 2,4,6-triisopropylbenzenesulfonyl chloride as key steps for 3′,5′-cyclization (Scheme 21) (Kim et al. 2012).

Also HDP-cP-AraC was designed as prodrug to treat PVR in rabbit, and the applied depot was visible for 6 months after a single intravitreal injection (Kim et al. 2012). In vitro release studies and intraocular in vivo drug depot visibility revealed that HDP prodrugs of cNMPs demonstrated significantly prolonged long-term release of cytarabine- and 5-F-2′-deoxyuridine metabolites, compared to a fast turnover of their corresponding HDP-5′-monophosphate prodrugs (Kim et al. 2012). In summary, the newly developed cNMP-HDP esters appear to have superior pharmacokinetic properties, ideally suited to reduce the frequency of uncomfortable intravitreal injections. It will be interesting to keep track of further medicinal developments of this lipid ester approach to deliver depot prodrugs for bioactive nucleosides and nucleotides, including cyclic nucleotides, for multiple therapeutic indications.



Scheme 20 Preparation of the cyclic nucleotide HDP-ester prodrug of 5-fluorouridine by phosphorylation and subsequent cyclization



Scheme 21 Preparation of the cyclic nucleotide HDP ester prodrug of cytarabine by phosphorylation and subsequent cyclization

7 Lipophilicity Determination and Membrane Permeability of cCMP, cUMP, and Analogs

In order to modulate intracellular signal transduction processes in vivo, the lipophilicity of a biologically active compound to be administered from the outside is of particular importance. The octanol/water partition coefficient log *P* is a generally accepted indicator for the expected capability of a given compound to pass through the cellular lipid bilayer by passive diffusion. Unfortunately, the determination of log *P* data is technically rather difficult for highly polar structures such as cyclic nucleotides. Therefore, lipophilicity information is often only obtained by fragment analysis and corresponding calculations (Mannhold et al. 2009). We used an HPLC method based on retention data on RP-18 reversed-phase silica during gradient elution (Krass et al. 1997), comparing a broad selection of analogs (http://www.biolog.de/technical-info/lipophilicity-data/). Instead of log *P*, the method yields the descriptor log k_g', ranking analytes according to their lipophilicity on a logarithmic scale as well. Since charged molecules like cyclic nucleotides have only negligible retention on reversed phases, ion pair chromatography with the lipophilic triethylammonium counter cation is used for analysis. According to these data, the lipophilicity of cNMPs increases with cCMP < cUMP < cGMP < cAMP, leaving cCMP as the least membrane-permeant analog which differs from cAMP by roughly a factor of 3 (Table 1). Unmodified cAMP itself (log $k_g' = 1.094$) is considered not to be membrane-permeant by passive diffusion, indicating that all other, even more polar endogenous cNMPs such as cUMP and cCMP will behave the same. An analysis of widely used cNMP analogs revealed that noteworthy diffusion into cells takes place only for analogs having a log k_g' of at least 1.2 (Genieser, unpublished results). This finding is in accordance with a study of Werner et al. using an immunoassay approach to measure membrane permeability of cNMPs (Werner et al. 2011). Only cNMP analogs with considerable hydrophobic modifications or lipophilic substituents, which counteract to the negative charge at the phosphate moiety, can be used for extracellular application. For example, the phosphorothioate modification of cAMP induces a moderately increased lipophilicity (log $k_g' = 1.210$ for the inhibitory Rp-cAMPS and log $k_g' = 1.320$ for the agonistic Sp-cAMPS). This increase is sufficient to improve membrane permeability, leading to widely used tools for the modulation of PKA pathways. By contrast, the same modification is expected not to be sufficient for cCMP (log $k_g' = 0.623$). Due to the polar cytosine nucleobase, Rp- and Sp-cCMPS (log $k_g' = 0.735$ and 0.801, respectively) still resemble a lipophilicity level below that of putatively non-permeant cAMP. Selecting suitable cCMP analogs for extracellular application has to consider the increased polarity of the pyrimidine nucleobase, and it is not surprising that there are only very few structures at hand, which could be used as membrane-permeant tools (Table 1). Interestingly, and for yet unknown reasons, the monobutyrylated 4-MB-cCMP with a log $k_g' = 1.743$ displays a high fractional log k_g' increase, leading to a similar lipophilicity compared to its purine-based adenine congener N^6-monobutyryladenosine-3′,5′-cyclic monophosphate (6-MB-cAMP, log

Table 1 Lipophilicity data for selected cCMP, cUMP, and related canonical cAMP and cGMP analogs

Cyclic nucleotide analog	log k_g'
cCMP	0.623
Rp-cCMPS	0.735
Sp-cCMPS	0.801
Rp-cCMPB	0.900
Sp-cCMPB	0.914
2′-O-MB-cCMP	1.509
4-MB-cCMP	1.743
MANT-cCMP	2.236
DB-cCMP	2.527
cUMP	0.692
MANT-cUMP	2.359
cGMP	0.774
2′-O-MB-cGMP	1.500
2-MB-cGMP	1.900
MANT-cGMP	1.999
DB-cGMP	2.560
cAMP	1.094
Rp-cAMPS	1.210
Sp-cAMPS	1.320
8-Br-cAMP	1.350
6-MB-cAMP	1.634
2′-O-MB-cAMP	1.927
DB-cAMP	2.420
MANT-cAMP	2.590

$k_g' = 1.634$). This anomaly is observed also with the dibutyrylated analog DB-cCMP and with cGMP analogs (DB-cGMP, 2-MB-cGMP).

DB-cCMP seems to be a useful tool with respect to permeability with a log $k_g' = 2.527$. However, the free butyrate released by endogenous esterases was described repeatedly to trigger own effects that might interfere with biological signaling of DB-cCMP (Soldatenkov et al. 1998; Crane 2000). This intracellular butyrate release is also relevant in experiments with other cyclic nucleotide analogs, including 2′-MB-cCMP (log $k_g' = 1.509$), which has a permeability comparable to 8-Br-cAMP (log $k_g' = 1.350$), a frequently used standard tool in cAMP research. Sodium butyrate or related butyrate-containing reagents are useful control reagents to test for potential side effects of butyrate in cell culture or tissues.

cCMP analogs that carry a prodrug construct at the cyclic phosphate, which masks the negative charge by means of an ester group (e.g., cCMP-AM), have considerably increased lipophilicity and improved membrane permeability.

Retention analysis of several different triester analogs seems to support the observation that the axial isomer is slightly more polar compared to the equatorial counterpart (compare Fig. 4). It would be of high interest to analyze the lipophilicity difference between a given charged cyclic nucleotide (e.g., cCMP) and its neutral triester version (cCMP-AM). However, a direct comparison of log k_g' lipophilicity data from charged and uncharged analogs is not entirely possible in this particular ion pair-based HPLC setting. The employed triethylammonium cation interacts exclusively with charged analytes, leading to artificially low log k_g'-values of the uncharged congeners. Although triester pyrimidine-based cNMP analogs would be suitable for log *P* determinations, no such studies were published to the best of our knowledge. Until the recent development of cUMP-AM and MANT-cUMP, there were no suitable analogs of cUMP commercially available, which could serve as molecular tools for extracellular applications. This situation has considerably hampered the evaluation of potential biological functions of this putative second messenger molecule. Especially in case of the polar pyrimidine cNMPs, the design of further improved analogs with lipophilic substituents is necessary. Depending on subsequent applications, either analogs with classical lipophilic substituents in the nucleobase and/or ribose or with versatile prodrug structures are required.

8 Conclusion and Future Perspectives

Early medicinal chemistry efforts to design analogs of the noncanonical pyrimidine cyclic nucleotides cCMP and cUMP mainly focused on the development of precursors for antitumor and antiviral nucleosides and nucleoside-5′-monophosphates, respectively. Apart from often tedious synthetic preparations, most of these analogs showed negligible pharmacologically relevant potencies. Furthermore, pyrimidine cNMP research itself suffered from scientific controversies about methodological approaches and subsequent artifacts, leading to flattened scientific activities for many years. After a revitalized interest in cCMP and cUMP in the early 2000s, many new cyclic nucleotide analogs became available in recent years. Improved large-scale cyclization procedures for cCMP and cUMP as starting nucleotides for subsequent syntheses were developed. New analogs with ω-amine-containing spacers, such as 4-AH-cCMP and 5-AA-cUMP, are universal precursors for the preparation of agaroses or for further derivatization with reporters like fluorescent dyes, spin labels, or biotin. All these tools are thought to be useful to identify and characterize new protein-binding partners of cCMP and cUMP, with ongoing studies and first reports already published. As soon as new and selective binding proteins have been identified, medicinal chemistry will concentrate on the design of cCMP and cUMP analogs with activatory or inhibitory properties for an in-depth characterization of involved biological pathways. Ideally, for such mechanistic tasks, a particular pyrimidine modification will be combined with a 3′,5′-cyclic phosphorothioate modification to avoid hydrolysis of the cyclic nucleotide and disturbing biological effects of released metabolites. In the field of

cAMP and cGMP, this approach led to superior tools with Sp-cNMPS analogs as activators and Rp-cNMPS analogs as inhibitors of corresponding receptor proteins. Both 2′-AHC-cCMP and 2′-AHC-cUMP are hydrolytically significantly more stable than their corresponding 2′-O-monosuccinyl progenitors, making them attractive starting molecules for the development of an improved generation of immunoassays with higher selectivity and sensitivity for the detection of endogenous cCMP and cUMP. To the best of our knowledge, an immunoassay for cUMP has not yet been described in the literature. cCMP-AM and cUMP-AM prodrugs were prepared and were found to be valuable tools to increase intracellular concentrations of cCMP and cUMP (Schwede and Seifert, unpublished results; Beckert et al. 2014). However, extracellular doses as high as 100–200 μM of cCMP-AM and cUMP-AM were required to generate biological effects, reflecting the need to further increase the lipophilicity of these prodrugs. One strategy to accomplish this task is to prepare the AM-ester prodrug of a hydrophobic cCMP analog like DB-cCMP. The resulting DB-cCMP-AM is expected to have membrane permeability comparable to DB-cAMP-AM, which in turn induced biological effects at an extracellular concentration of 10 μM or below (Schultz et al. 1993; Schwede et al. 2015). Additional alternatives, such as acyloxymethyl prodrug groups with superior lipophilicity, like butyroxymethyl ester (BM), pivaloxymethyl ester (POM), or 4-acetoxybenzyl ester (pAB) should be taken into consideration for the design of next-generation prodrugs of cCMP and cUMP as well. This strategy is of special interest for cUMP prodrugs, since for obvious structural reasons, dibutyrylated cUMP is not existent. Albeit not intended as prodrugs for the investigation of second messenger properties of cCMP and cUMP, the further development of 5-F-2′-deoxy-cUMP-HDP and ara-cCMP-HDP as long-term depots and preclinically most advanced pharmacophores will be of particular interest in the coming years.

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Holistic Methods for the Analysis of cNMP Effects

Manuel Grundmann and Evi Kostenis

Contents

Abstract

Cyclic nucleotide monophosphates (cNMPs) typify the archetype second messenger in living cells and serve as molecular switches with broad functionality. cAMP and cGMP are the best-described cNMPs; however, there is a growing body of evidence indicating that also cCMP and cUMP play a substantial role in signal transduction. Despite research efforts, to date, relatively little is known about the biology of these noncanonical cNMPs, which is due, at least in part, to methodological issues in the past entailing setbacks of the entire field. Only recently, with the use of state-of-the-art techniques, it was possible to revive noncanonical cNMP research. While high-sensitive detection methods disclosed relevant levels of cCMP and cUMP in mammalian cells, knowledge about the biological effectors and their physiological interplay is still incomplete. Holistic

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biophysical readouts capture cell responses label-free and in an unbiased fashion with the advantage to detect concealed aspects of cell signaling that are arduous to access via traditional biochemical assay approaches. In this chapter, we introduce the dynamic mass redistribution (DMR) technology to explore cell signaling beyond established receptor-controlled mechanisms. Both common and distinctive features in the signaling structure of cCMP and cUMP were identified. Moreover, the integrated response of whole live cells revealed a hitherto undisclosed additional effector of the noncanonical cNMPs. Future studies will show how holistic methods will become integrated into the methodological arsenal of contemporary cNMP research.

Keywords
cCMP • cUMP • DMR • Label-free • Noncanonical cNMP

1 Introduction

The cyclic nucleotides cAMP and cGMP are well-established second messenger molecules. In contrast, noncanonical cyclic nucleotide monophosphates (cNMPs) such as cCMP or cUMP only play a niche role in the family of intracellular signaling molecules and were for a long time far from being recognized as true second messenger. A molecule to be considered as such must fulfill several criteria (Hardman et al. 1971; Schultz and Rosenthal 1985; Gao and Vanhoutte 2014). In essence this is the quest for (1) a generating system upon stimulation with a first messenger and (2) a cellular effector system that controls (3) a biological function in cells, organs, or tissues. To regulate the second messenger effect, a (4) signal-terminating system must be present. Finally, (5) membrane-permeable analogs of the second messenger or often bacterial toxins increase the intracellular level of the second messenger and mimic the biological response.

Despite compelling evidence for the existence of noncanonical cNMPs already early in the history of cell biology research, the exploration of cCMP and cUMP has never been straightforward. Strikingly, methodological issues compromised the acknowledgment of noncanonical cNMPs. For example, the postulation of a specific cCMP-generating enzyme turned out to rely on an artifact (Cech and Ignarro 1977; Gaion and Krishna 1979). The field was further confronted with infidelity of experimental tools such as the cross-reactivity of detecting antibodies that were used to identify cNMP entities (Anderson 1982). These findings disqualified immunologic techniques as an experimental approach to study cCMP biology. In addition, early mass spectrometry (MS) methods failed to accurately identify and quantify cNMP level, due to technical limitations of fast-atom bombardment MS, which was insufficient in both sensitivity and precision to analyze cNMPs in complex biological matrices (Newton et al. 1984). Furthermore, radiometric analysis of cCMP and cUMP production can only be tracked in cell-free systems (Seifert and Dove 2013), and HPLC methods without implemented mass spectrometric

analysis are confined to application with unphysiologically high amounts of the regarding cNMPs and could thus not be applied to intact cells (Göttle et al. 2010). This limited the biological relevance of data generated using these techniques. All together, these methodological drawbacks led to a negative perception of cCMP and cUMP research and a mistrust in this field within the scientific community. This might explain why noncanonical cNMPs have long been underappreciated as second messenger molecules with distinct biological functions. Evidently, the biological role of noncanonical cNMPs was largely enigmatic because research was mainly focused on cAMP/cGMP and their function.

Recently, a revival of research activities in the field of noncanonical cNMPs using state-of-the-art experimental techniques (HPLC-MS/MS, HPLC-MS/TOF, and DMR) and key experimental tools (cNMP-AMs and ExoY) could unequivocally identify cCMP and cUMP as true second messengers (Seifert 2015). Overall, the field of cCMP and cUMP research was given a strong boost by technological advances. In this chapter, we introduce a label-free technique as a new component in the scientist's toolbox to investigate the biological effects of cyclic nucleotides. We give an overview on the current and possible future state of this methodologic approach by discussing the advantages and disadvantages of this technique to analyze cellular signal transduction.

2 Measurement Principle and General Implications for Assay Design

Dynamic mass redistribution (DMR) is measured by an optical biosensor and represents a relatively new approach to address biomedical research questions (Wang et al. 1990). The first applications of DMR focused on the study of protein–protein interaction using immobilized target molecules (Myszka 1999). Over time, the method was subsequently extended to cell-based applications, which thus made previously unresolved research questions become accessible (Fang et al. 2006; Lee et al. 2008). It provides a holistic view on processes at the whole cell level without the need of tagging proteins of interest risking to perturb natural cellular behavior (label-free) (Kenakin 2010; Rocheville and Jerman 2009; Scott and Peters 2010). The real-time unbiased perspective broadens the viewing angle on cellular processes and entails a reduced risk missing cellular signaling events in both time and space.

The principle of DMR relies on the phenomenon of resonant waveguide grating, in which light of a certain coupling wavelength that corresponds to the physical properties of the biosensor and adjacent material is guided through the sensor and thereby generates an evanescent field that reaches approximately 150 nm into the space above the biosensor (Grundmann and Kostenis 2015; Schröder et al. 2011). Figure 1 illustrates the detection principle and depicts relevant parameters. In a typical DMR assay, cells grow on or are in close proximity to the sensor in the case of suspension cells and thus directly affect the physical properties of the biosensor. Changes in the optical density within the range of the evanescent field lead to

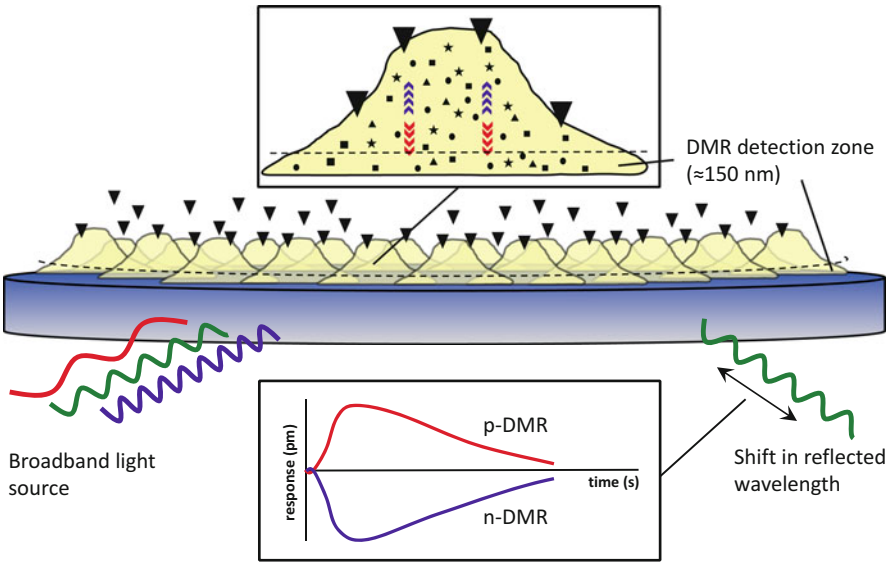

Fig. 1 Measurement principle of dynamic mass redistribution (DMR). Broadband light illuminates the bottom of a waveguide-grated biosensor. A specific wavelength is then propagated through the sensor and induces an evanescent field with a range of approximately 150 nm above the sensor. The wavelength of the reflected light is determined by the optical density above the sensor. If more mass is within the range of the biosensor, the light energy decreases (longer wavelength, p-DMR), whereas mass redistribution away from the sensor increases the energy (shorter wavelength, n-DMR). The difference of the reflected wavelength (in pm) are then plotted against the time (in s) (Grundmann and Kostenis 2015)

energy changes of the coupled optical wave, which in turn is detected by a shift of the reflected wavelength. This physically complex process relies on a rather simple biophysical event, i.e., the change of optical density above the biosensor and thus in the cell layer (Fang et al. 2006). All fundamental biological cellular events such as proliferation, differentiation, migration, adherence, or cell death involve structural changes within the cells that are caused by rearrangement of intracellular particles or within the cytoskeleton. In turn, most if not all reactions of a cell can be monitored as a response on a morphological level.

This universal readout allows the detection of an integrated cell response representing cellular behavior as a total. The versatility of this detection principle in combination with its wide range of sensitivity allows the application of genetically engineered cells, native cells, and primary cells that endogenously express the target protein of interest (Grundmann and Kostenis 2015; Schröder et al. 2011). But this detection principle entails also a major drawback of the method, which is the complexity of the assay output. The challenge of interpreting DMR data is to decipher as what is represented in the optical traces obtained by the structural changes within the cells. To achieve this goal, several experimental approaches have been established. Choosing an appropriate cell system according to the

research question provides the basis for meaningful data. Especially the question of relevant negative controls can be answered by selecting cells with a suitable genetic background such as lack of the target protein of interest. In case of endogenously expressing cell samples, knockout or siRNA approaches may assume that role. Provided the availability of high-quality tool compounds, pharmacological perturbation by (ant)agonists or stimulators/inhibitors helps to interrogate the involvement of specific signaling partners that contribute to the overall cell response circumventing the necessity of genetic manipulation (Grundmann and Kostenis 2015; Schröder et al. 2010; Blättermann et al. 2012; Hennen et al. 2013).

Due to its versatility, the DMR assay is therefore suited as both primary assay for target validation and evaluation of off-target effects as well as an orthogonal assay format for mechanistic studies on compound biology. The DMR method has proven very useful in both academia and industry especially in the field of G protein-coupled receptors (GPCRs) which comprise the largest group of drug targets today (Scott and Peters 2010; Kenakin 2009, 2010). Particular interest has been focused on the phenomenon of functional selectivity, also referred to as signaling bias. Depending on ligand nature and receptor constitution, GPCRs can process signaling input differentially, i.e., some signaling routes are preferentially activated over others (Kenakin and Christopoulos 2013). The unbiased DMR technology provides an important means to elucidate multifaceted signaling networks within living whole cells in real time. However, as this experimental approach is not limited to cell surface receptors, further applications at the *post*-receptor level are equally well conceivable. Although principally suited, to date, the direct effects of intracellular second messenger molecules have only been addressed rarely with this method (Beckert et al. 2014). In this chapter, we demonstrate the applicability of label-free techniques to the field of noncanonical cyclic nucleotide research.

3 Noncanonical Cyclic Nucleotides in Mammalian Cells

It could be shown that in comparison to other domains of life, cUMP and cCMP are mainly present in mammalian cells suggesting that from an evolutionary perspective cUMP and cCMP represent a relatively young group of substances (Hartwig et al. 2014). This might indicate that these molecules undertake a more specialized task in the signaling network of mammalian cells. Notably, high levels of cUMP and cCMP have been found in HEK293 and B103 neuroblastoma cells, as well as in primary astrocytes and epithelial cells (Hartwig et al. 2014). Of interest, most cCMP and cUMP levels were thus measured in cells with neurological origin and highly specialized cell types. Because the signaling machinery in this cellular environment seems to be adequately adapted to interact with cCMP and cUMP, these cell systems might be best suited as a starting point to investigate the biological effects.

4 Effect of cNMP Modulating Agents in HEK293 Cells

cAMP is a ubiquitously prevalent and well-described second messenger (Gancedo 2013). Therefore we probed changes of intracellular cAMP levels as a prototype cNMP effect in label-free DMR assays. Since GPCR-mediated cell signaling is overridingly explored and evaluated by this method, we stimulated HEK293 cells with isoproterenol, an agonist at the β-adrenergic receptor. This model class A GPCR is coupled to Gαs proteins and leads to stimulation of the adenylyl cyclase (AC), which in turn increases intracellular cAMP levels. Further signaling of cAMP (also referred to as cAMP-dependent pathway) can occur via activation of protein kinase A (PKA) (Krebs 1989), but also the protein kinase G (PKG) (Eckly-Michel et al. 1997; Burnette and White 2006), via interaction with Epac (exchange protein directly activated by cAMP) (Rehmann et al. 2007) or activation of cyclic nucleotide-gated ion channels (Biel 2009) and presumably other effectors. cAMP is positioned in the center of this signaling cascade and plays a key role in mediating a cell response. Modulating intracellular levels of this second messenger consequently leads to a cell response that is compiled as a morphological change and subsequently captured by the DMR readout. Figure 2a gives an overview of the distinct interaction points of the cAMP-modulating agents. It should be stressed that the resulting DMR response following isoproterenol stimulation is an amalgam of different signaling events that merge at the level of cellular rearrangement (Fig. 2b). Signaling upstream or in parallel of cAMP can therefore similarly contribute to the overall cell response. However, since the occurrence of cAMP is crucially linked to the signaling mechanism as well as the physiological function of the β-adrenergic receptor, this second messenger plays a decisive role in the cellular response.

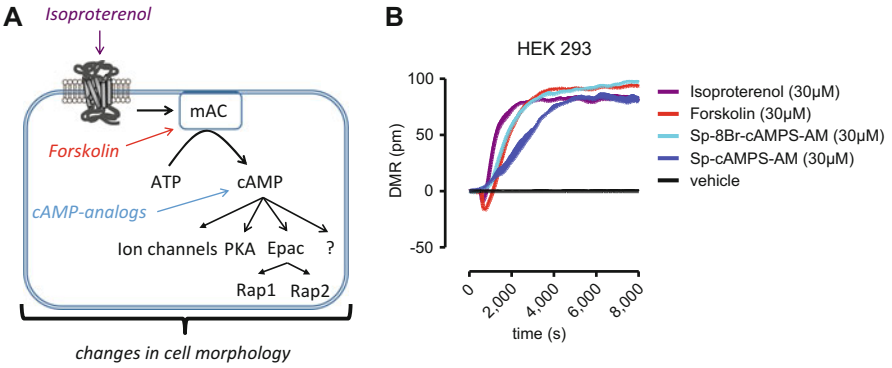

Fig. 2 (**a**) Schematic of cAMP signaling. Changes in intracellular cAMP level can be achieved by either targeting surface receptors such as the Gαs-coupled β-adrenergic receptor with isoproterenol, the membranous adenylate cyclase (mAC) with the diterpene forskolin, or direct elevation by synthetic cAMP analogs. As a response several effectors are involved that ultimately lead to a change of cell morphology. (**b**) Dynamic mass redistribution of native HEK293 cells is captured upon stimulation with various compounds affecting intracellular cAMP levels. Note the different kinetics upon compound addition at 5 min. Shown are representative traces in triplicates + s.e.m.

A more direct approach to higher intracellular cAMP levels is the stimulation of the membranous adenylyl cyclase (mAC). This can be achieved by incubating the cells with the diterpene forskolin, a natural product of the Indian Coleus plant. After passing the cell membrane, this compound acts intracellularly at the mAC isoforms 1–8 (but not isoform 9) and can induce cAMP production dose dependently (Fig. 2b) (Seamon and Daly 1986). To introduce yet another possibility to mimic effects of cAMP, we applied cell membrane-permeable cAMP analogs in the aforementioned HEK293 cell system. We measured the effects of two closely related cAMP analogs, which showed variances in their lipophilicity and finally compared them with isoproterenol and forskolin (Fig. 2b). All substances induced a robust positive DMR response although different signal kinetics were observed. Isoproterenol, acting at a cell surface receptor, showed the earliest cell activation directly after compound addition. After a transient negative DMR, a durable and long-lasting positive DMR signal prevailed the response. Forskolin induced a qualitatively congruent but temporally shifted DMR trace. This indicates a kinetic difference in the cellular activation process between targeting a cell surface receptor and the downstream effector system. Also the cAMP analog-induced DMR with a delay in the onset of the signal, thus overcoming the cell membrane barrier, appears to be a time-consuming step in the activation process. The 8-bromo substituent at the cAMP analog rendered the molecule more lipophilic and displayed faster kinetics compared to the unsubstituted derivative (Fig. 2b). Moreover, the lack of initial negative DMR response directly after compound addition in the case of the cAMP analogs might indicate a role of the AC activity in the process of cellular rearrangement, as the negative shift is more pronounced after stimulation with the direct mAC activator forskolin and the Gαs-coupled receptor agonist isoproterenol (Fig. 2b).

5 Validation of cNMP Level-Manipulating Compounds by DMR

A biological mimicry by toxins that induce cNMP production is a characteristic of bona fide second messenger. Raising the intracellular level of the second messenger and subsequent observation of a cellular effect gives the opportunity to gain insight into the function and significance of a specific second messenger molecule. As delineated in Fig. 2, there are different ways to achieve an intracellular rise of a second messenger molecule. The most immediate approach is the direct application of the second messenger onto the cells leading to a biological effect in some cases (Silos-Santiago et al. 2013). The noncanonical, unmodified cyclic nucleotides cCMP and cUMP were therefore examined for their capacity to induce a cell response in native HEK293 cells from the cell surface. Those signaling events could potentially interfere with signaling events generated from intracellular sources and complicate the decoding of the DMR response. These considerations are especially important under the light of previous works reporting a biological effect of cNMPs exerted from the extracellular milieu. The first messenger role of

certain cyclic nucleotides such as cGMP is well documented by experimental data (Bieck et al. 1969; Salzman and Levine 1971; Bloch et al. 1974). Indeed, cNMPs can be transported between cells, which enable the possibility to act via cell surface targets (Schneider and Seifert 2015). cAMP, for example, can function as a "pro"--first messenger, since metabolites such as adenosine and adenine are able to induce signaling via the activation of distinct GPCRs (Jackson and Raghvendra 2004; Russel et al. 2008; von Kügelgen et al. 2008). Large numbers of uncharted but potential structures, such as many orphan receptors, might also be targets for unmodified cyclic nucleotides. Indeed, a specific GPCR for cCMP has already been suggested (Chen et al. 2014). However, in the case of HEK293 cells, there are apparently no endogenously expressed surface receptors that are targeted by unmodified cCMP and cUMP as illustrated by a lack of cell response (Fig. 3a). On the other hand, membrane-permeable cNMP analogs that mimic cNMP activation patterns are an elegant means to investigate their second messenger role. Acetoxymethyl ester of cCMP and cUMP, cCMP-AM and cUMP-AM,

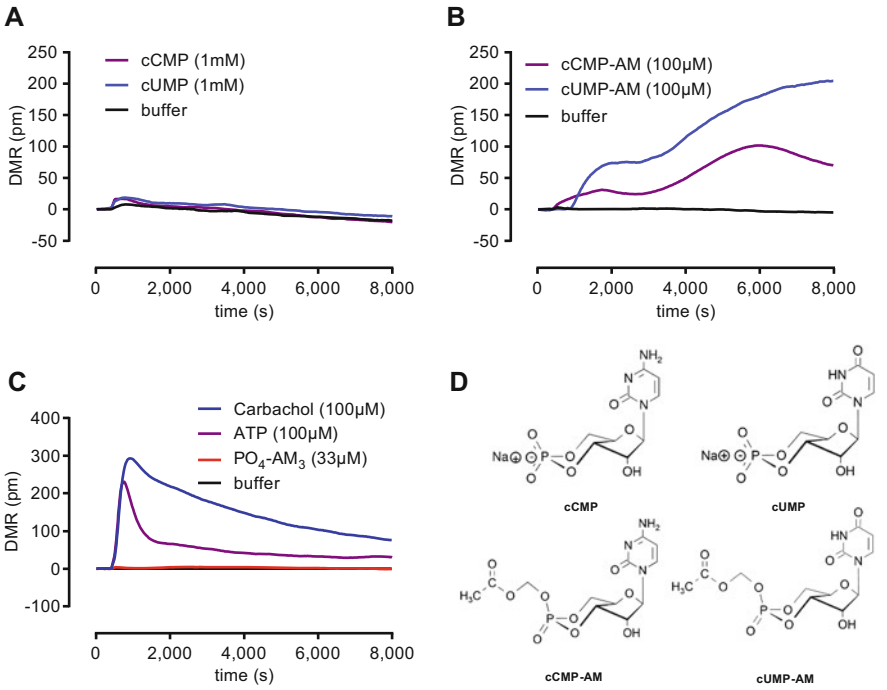

Fig. 3 (**a**) Unmodified cyclic nucleotides show no activity in the DMR assay indicating a lack of cNMP surface receptors in native HEK293 cells. (**b**) Membrane-permeable acetoxymethyl esters of cognate cyclic nucleotides induce a robust HEK293 cell response. (**c**) Carbachol and ATP activating endogenously expressed GPCRs serve as viability control, while corresponding amounts of the acetoxymethyl phosphate esters show no activity in the DMR assay. (**d**) Chemical structures of cUMP, cCMP, cUMP-AM, and cCMP-AM. (**a–c**) Shown are representative traces in triplicates as mean value

respectively, showed substantial activity in the DMR assay (Fig. 3b). This chemical modification confers plasma membrane permeability onto the substances thereby enabling interaction with intracellular effectors after in situ cleavage of the ester bond by cytosolic esterases (Schultz et al. 1994). Importantly, in native HEK293 cells, the acetoxymethyl ester itself is biologically inert as corroborated by the lack of effect in the holistic biosensor readout. ATP and carbachol, two well-established GPCR activators, however, demonstrated proper cell viability and responsiveness (Fig. 3c). A comparison of the chemical structures involved in this process is given in Fig. 3d. It can easily be perceived that the AM ester increases compound lipophilicity and thus enables the structures to permeate cell membranes, whereas the non-modified cyclic nucleotide are too polar to cross the lipid bilayer. However, at this point, it should be taken into consideration that even hydrophilic cNMPs might be taken up into cells by transport mechanisms (Li et al. 2012; Chen et al. 2014). Nevertheless, the in situ release of the functional and genuine cyclic nucleotide at an important site of second messenger effects, i.e., the cytosol, is a major advantage over other cNMP derivatives such as halogenated cyclic nucleotides, for which alterations of the biological behavior cannot be ruled out.

6 Cyclic Nucleotide-Induced Cell Response

A conspicuous challenge of DMR readouts is the interpretation of the results. Complexly composited signal structures are difficult to decipher without further examination since the readout is not simplistically associated with a certain signaling event. The holistic nature of the DMR assay reflects the cellular events on multiple levels at the same time and captures the sum of the processes in a measure that can change in only two ways: positive or negative DMR (although the temporal resolution is high, so that qualitative distinctions based on kinetic differences are accessible) (Beckert et al. 2014). This difficulty is illustrated in Fig. 4 showing increasing concentrations from 10 up to 300 μM of cGMP-AM- and cCMP-AM at HEK293 cells. Clearly, the cells do not react homogenously to a cNMP stimulus over the full range of concentrations. While showing a dose-dependent increase in DMR up to 50 μM for the guanine nucleotide, higher concentrations evoked clearly different shapes of optical traces in both regards, kinetics and amplitude (Fig. 4a). It is evident from the data that at least two opposing events interfere in the signature with one tending to positive DMR and another to negative DMR responses. Depending on the concentration, one effect prevails the other in the phenotypic sum of events (red/purple vs. blue traces in Fig. 4a). This phenomenon can also be seen for cCMP-AM although in different characteristic changes and at slightly different concentrations (Fig. 4b). While up to 50 μM cCMP-AM induces a nearly linear effect trace over the time, the signal becomes sluggish at 100 μM and finally entirely changes its shape at 300 μM. Hence, it is very important to pay attention to the concentration used in mechanistic studies on the biological effects of cNMPs to assure a homogenous DMR activation as the basis for further investigations.

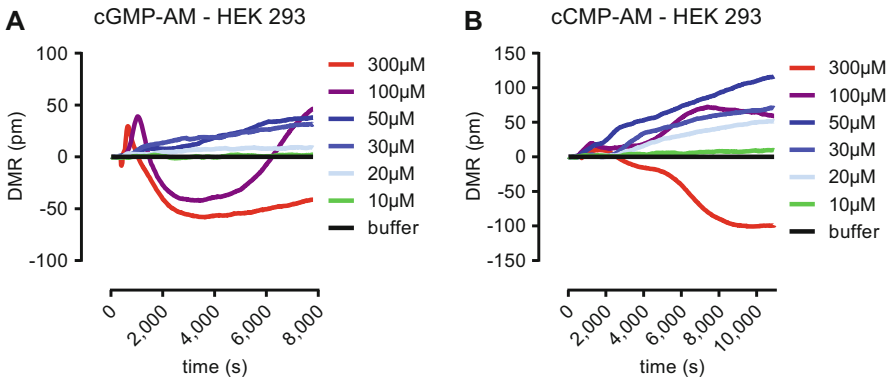

Fig. 4 Comparison of DMR traces of HEK293 cells stimulated with various concentrations of cGMP-AM (**a**) or cCMP-AM (**b**). Shown are representative traces in triplicates as mean value

7 Experimental Validation of Signaling Pathway Deconvoluting Tool Compounds

The multilayered character of a holistic whole cell readout raises the question which and to what extent certain signal events are involved in the cellular phenotype typified in the DMR traces. Those in-depth mechanistic studies are only feasible by selective inhibition of distinct components along the signaling pathway to decipher the DMR readout. This was previously demonstrated for G protein-coupled receptor signaling by several studies (Schröder et al. 2010; Blättermann et al. 2012; Hennen et al. 2013; Verrier et al. 2011) but is rather uncharted territory for signal transduction analysis at the *post*-receptor level (Beckert et al. 2014).

Since classic cyclic nucleotide second messengers are known to interact with protein kinases, such as protein kinase A and G and PKA and PKG, respectively (Krebs 1989; Glass and Krebs 1980), we probed the capability of cCMP to signal via these kinases. cAMPS and Rp-8-Br-cAMPS were used as inhibitors for PKA (Gjertsen et al. 1995), while Rp-8-Br-PET-cGMPS and Rp-8-cCPT-cGMPS served as PKG inhibitors (Butt et al. 1994, 1995; Poppe et al. 2008). Since the inhibitors slightly differ in their preference for blocking distinct kinase subunits, we aimed to combine two protein kinase inhibitors to maximize the inhibiting effect and cover the full activation spectrum of these signaling proteins. Due to the holistic nature, DMR assays are well suited to detect off-target effects of probe compounds. Because even the highest concentration of both PKA inhibitors did not show any effects on their own, we ruled out any intrinsic activity that could potentially superimpose with the specific cNMP effects (Fig. 5a,b). In consequence, we combined 500 µM cAMPS and 500 µM Rp-8-Br-cAMPS for further experiments (hereafter referred to as PKA combo). For the PKG inhibitors, only 500 µM of Rp-8-Br-PET-cGMPS itself induced a cell response; hence, 300 µM of this inhibitor was subsequently chosen to cooperate with 500 µM of Rp-8-cCPT-cGMPS to

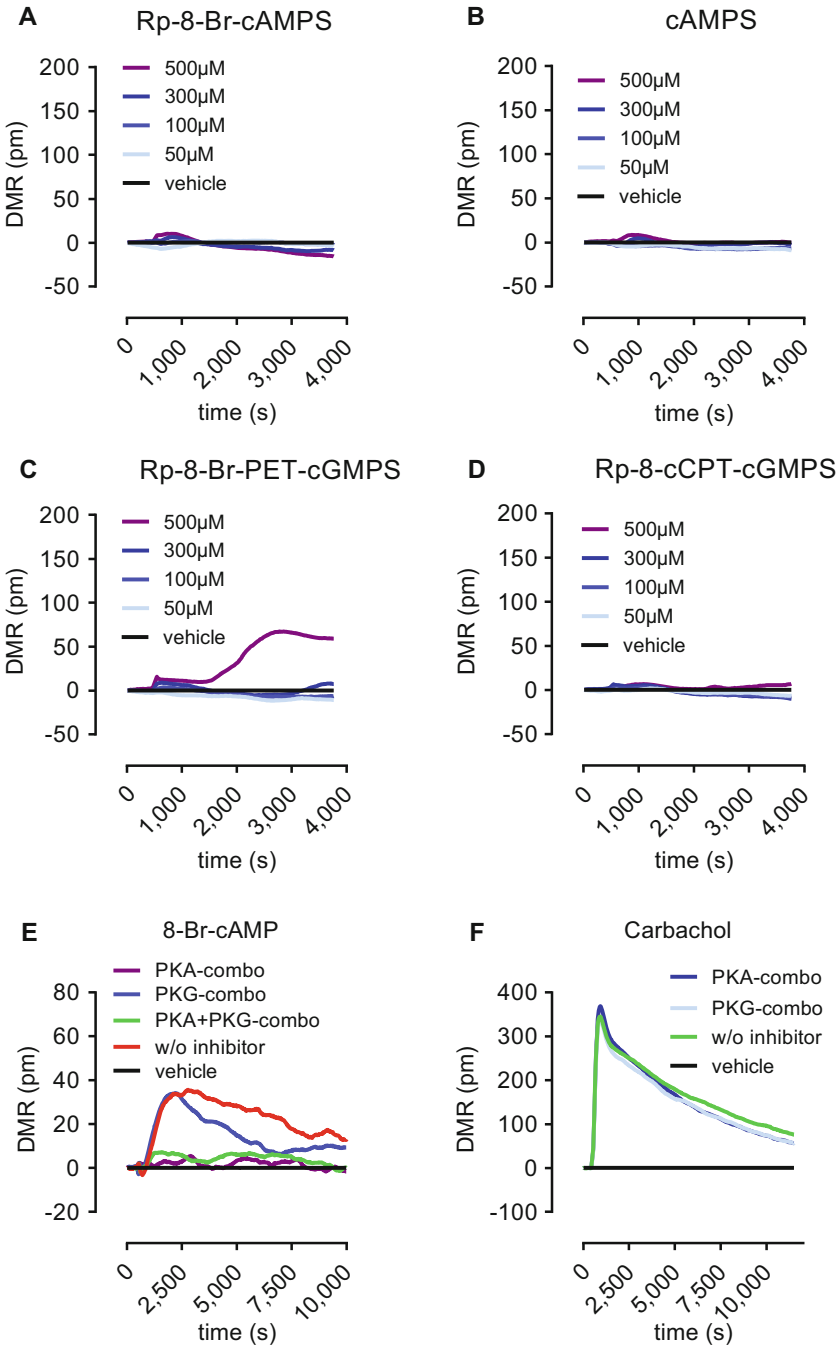

Fig. 5 Characterization of protein kinase inhibitors in the DMR assay. Lack of cell response to the protein kinase A (PKA) inhibitors Rp-8-Br-cAMPS (**a**) and cAMPS (**b**). Protein kinase G (PKG) inhibitor Rp-8-Br-PET-cGMPS induces a DMR response only in the highest concentration

build the PKG inhibitor combination (PKG combo) (Fig. 5c,d). The fidelity of the inhibitors was tested using the well-known PKA-activator and cAMP analog 8-Br-cAMP revealing that the PKA combo and the PKA-PKG combination (PKA-PKG combo), but not the PKG combo, silenced 8-Br-cAMP-induced cell response (Fig. 5e). In addition, the protein kinase inhibitors did not affect carbachol-induced muscarinic acetylcholine receptor signaling in HEK293 cells, which further corroborates target specificity of these tool compounds acting as selective protein kinase inhibitors (Fig. 5f). In the following, we put these pharmacological tools to use to decode the cellular cNMP effect.

8 Mechanistic Studies of Cellular cNMP Effects

As the previous results already indicated, the signature of cells displaying either cAMP, cGMP, cCMP, or cUMP signaling differs at first glance (compare Fig. 3c, Fig. 4, and Fig. 5e). To further examine this consideration, several PK inhibitor combinations were used to scrutinize cNMP-derived optical traces. The cell response upon stimulation with 10 μM cCMP-AM was completely silenced in the presence of protein kinase A inhibitors, suggesting a key role of this effector in the signaling of cCMP (Fig. 6a). However, this effect was overcome by an increased cCMP-AM concentration (30 μM) as revealed by the restoration of a delayed positive DMR response in the presence of PKA inhibitors, which indicates a concentration-dependent and competition-like interaction of the components in the signaling machinery (compare purple trace in Fig. 5a with Fig. 6b). Remarkably, PKG inhibition resulted in the disclosure of a rapid activating cellular event after cCMP-AM stimulation, which was maintained in the presence of PKA-PKG inhibitor combination (Fig. 6a,b). Thus, cCMP engages signal transduction via PKA and PKG and at least one additional target that emerges as a rapid cellular rearrangement. Notably, this target is under prioritized control of PKG rather than PKA. This notion is supported by the observations of increased c-fos gene expression in the presence of PKA and PKG inhibition, demonstrating a PKA-PKG-independent effect (Beckert et al. 2014) and the apoptosis-inducing effect of cCMP on S49 lymphoma cells in a PKA-PKG-, Epac-, and HCN2/HCN4 channel-independent fashion (Wolter et al. 2015).

cGMP-AM was used to study cGMP signaling in the DMR assay. By specifically rising intracellular levels of the second messenger cGMP, we observed characteristic changes of the cellular morphology. After an initial and transient increase of DMR, the signal drops to lower levels, from where it slowly increases (Fig. 6c).

Fig. 5 (continued) (500 μM) (**c**), while Rp-8-cCPT-cGMPS shows no effect (**d**). (**e**) Proper function of PKA inhibitors and exclusion of unspecific inhibition by PKG inhibitors is illustrated by interrogation of HEK293 cell response upon the PKA-activator 8-Br-cAMP with the protein kinase inhibitors, either alone or in combination. (**f**) Specificity of protein kinase inhibition is confirmed by lack of effect on carbachol-induced control traces. Shown are representative traces in triplicates as mean value

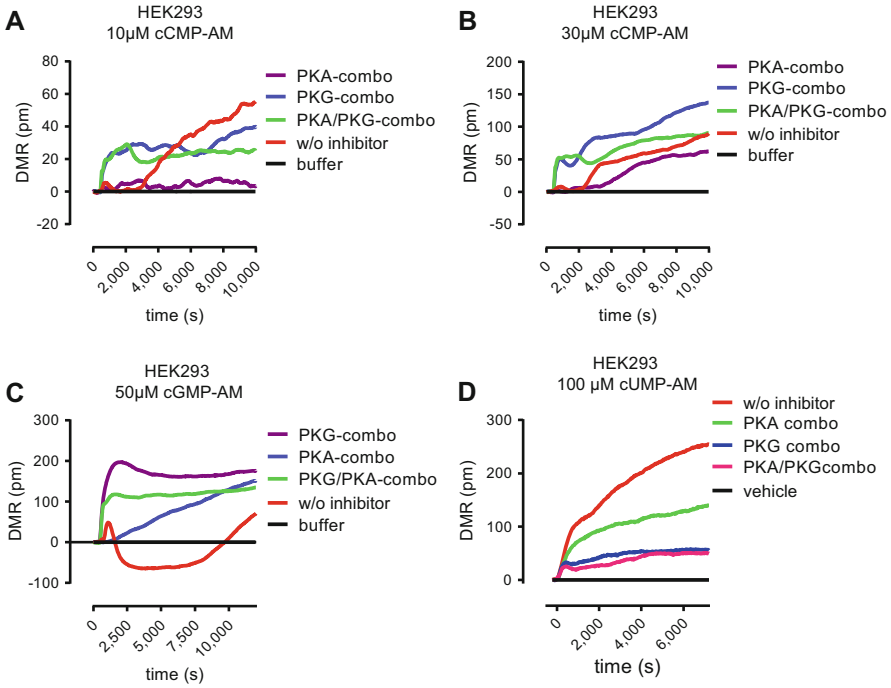

Fig. 6 Effect of PKA, PKG, or PKA-PKG inhibitors on HEK293 cells stimulated with either (**a**) 10 µM or (**b**) 30 µM cCMP-AM. Effect of protein kinase inhibitors on (**c**) cGMP-AM- or (**d**) cUMP-AM-induced HEK293 cell response. Shown are representative traces in triplicates as mean value

Despite a difference between the unperturbed traces, cGMP-mediated signaling was shown to be regulated by similar mechanisms as cCMP. The prominent role of protein kinase G in the cGMP is adequately described and corroborated by the whole cell assay. In the cases of both cCMP and cGMP signaling, the label-free assay revealed a crucial role of protein kinase G particularly in the control of early cell activation. Moreover, PKA could also be confirmed to modulate cGMP signaling as stated by others (Fig. 6c and Jiang et al. 1992; Wörner et al. 2007). Under the impression of the increasingly acknowledged concept of signal transduction as a network structure instead of linear pathways, a cross talk between PKA- and PKG-dependent signal hubs seems likely and was posed in previous works (Eckly-Michel et al. 1997; Burnette and White 2006) and a cross-reactivity of cAMP at PKG and cGMP at PKA, respectively, was shown by others (Jiang et al. 1992; Wörner et al. 2007; Eckly-Michel et al. 1997; Burnette and White 2006). A crystal structure of PKG bound to cAMP was resolved by Kim et al. underlining a certain degree of signaling promiscuity of cNMPs (Kim et al. 2011).

The analysis of cUMP-generated cell activation disclosed both parallels and discrepancies with cCMP. The natural response to cUMP displayed a different

kinetic compared to cCMP (Fig. 6d). This might by be owed to the fact that cCMP and cUMP are differently degraded by phosphodiesterases (Reinecke et al. 2011). Thus, the different lifespan of the biologically active elements might cause distinct kinetics in the cellular phenotype, which is detected by the DMR technology. On the other hand, parallel to cCMP, both protein kinases (PKA and PKG) and additional signaling partners are involved in cUMP-mediated cell response (Wolter et al. 2011). However, the DMR assay disclosed an interplay of these signaling partners, which is distinct from that of cCMP. This is not only embodied in the unperturbed cell signature, which portrays a rather uniform kinetic in the change of cell morphology for cUMP in comparison to cCMP, but got further evident with the help of the protein kinase inhibitors (Fig. 6d). In contrast to cCMP and cGMP, PKG does not regulate an early cell response of cUMP, but rather PKA and PKG showed a shared but commutated inhibiting impact on cUMP signaling. However, in accordance with the results derived from cCMP and cGMP, also cUMP signaling uses capacities beside protein kinases A and G. Since cUMP was shown to activate HCN channels 2 and 4 (Zong et al. 2012), it might be a future step to elucidate HCN channel activation in the DMR assay. Further evidence for a difference in target selectivity for cUMP versus cCMP was provided by data suggesting that cUMP but not cCMP activates Ca^{2+}-activated cation channels in rat insulinoma cells (Reale et al. 1994).

9 Further Insights into cCMP Signaling

A focus on the temporal shift in the cCMP trace in the presence of the protein kinase inhibitors revealed a concentration-dependent effect and thus confirmed a target specificity of cCMP signaling kinetics. HEK293 cell response upon cCMP stimulation was recorded in the presence of low and high concentrations of the PKA and PKG inhibitors, respectively. Increasing PKA-inhibitor concentrations delayed the onset of the DMR signal, whereas increased PKG inhibition shifted the signal commencement to earlier time points (Fig. 7a). These observations corroborate the opposing impacts of two key signaling units on the phenotypic cell response to cCMP.

cAMP regulates signaling via two receptors, i.e., the protein kinase A or cAMP-dependent protein kinase and the exchange proteins directly activated by cAMP (Epac) or cAMP-regulated guanine nucleotide exchange factors (cAMP-GEF). Since Epac proteins are well-defined targets of the common cAMP signaling pathway (Rehmann et al. 2007), we examined the ability of cCMP to signal via Epac. By overstimulating Epac with 300 μM 8-pCPT-2'-O-Me-cAMP, we mask the ability of Epac to contribute to the overall signaling of cCMP (Poppe et al. 2008; Holz et al. 2008). In contrast to cAMP, cCMP-mediated cell activation does not involve Epac as we could not detect a significant difference in the cCMP response between cells that were unable to signal via Epac (prestimulated with Epac activator) and untreated cells (Fig. 7b).

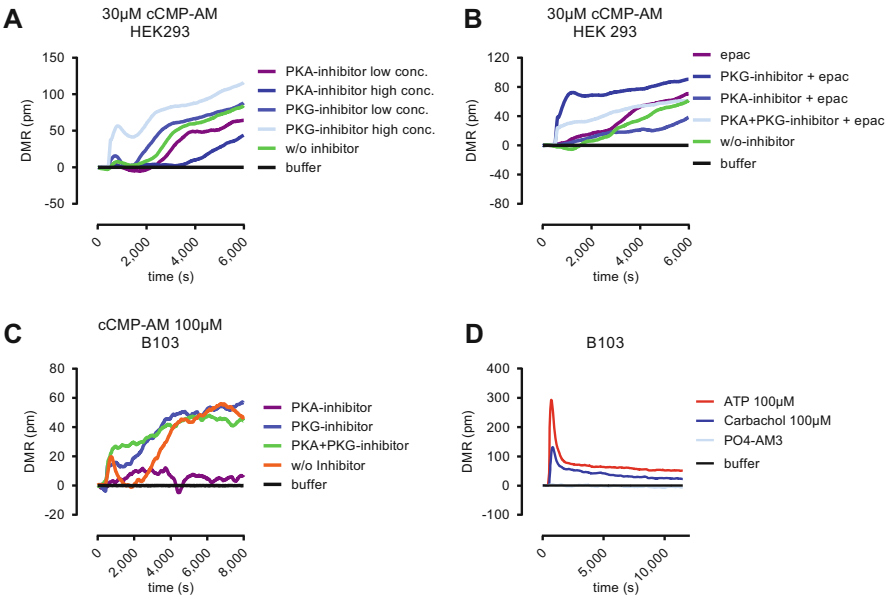

Fig. 7 (**a**) Concentration-dependent effect of PKA and PKG inhibitor on the kinetics of cCMP-mediated cell response. (**b**) DMR record of cCMP-activated HEK293 cells that were prestimulated with either the Epac activators and/or protein kinase inhibitors. (**c**) DMR response of B103 neuroblastoma cells stimulated with cCMP-AM in the absence and presence of protein kinase inhibitors. (**d**) ATP and carbachol, but not PO_4-AM_3, induce robust B103 neuroblastoma cell response. Shown are representative traces in triplicates as mean value

A major advantage of the DMR assay is the applicability to various cell lines, since no labels or genetic engineering techniques are needed. B103 neuroblastoma cells contain surpassing amounts of cCMP and cUMP levels and therefore raise interest in the function of the noncanonical cNMPs in this highly specialized biological environment. Analysis of cCMP signaling interactions in B103 cells corroborated the combined involvement of protein kinase A and G for cCMP as seen in the HEK293 cell background (Fig. 7c). However, while PKG inhibition in HEK293 disclosed an early cell activation, this rapid cell activation upon cCMP stimulation was always apparent in B103 but was rather sustained in the presence of the PKG inhibitors, whereas PKA inhibition silenced the entire cell response. The overall similarity of the cell response signatures and the sensitivity to specific signaling pathway inhibitors between HEK293 and B103 neuroblastoma cells indicate a common signaling pattern for cCMP, although the contribution of certain signaling elements seems to vary across the cellular background. Signal specificity was confirmed by control experiments demonstrating no cell activity upon addition of the negative control PO_4-AM_3 but proper cellular responsiveness to GPCR signaling by carbachol and ATP (Fig. 7d).

10 Concluding Remarks and Future Perspectives

A special feature of the label-free DMR technology is the capability to reflect the cellular phenotype in an unbiased fashion. Yet, it is possible to selectively recognize distinct signaling events with the help of pharmacological tool compounds or molecular biology approaches. The identification of the effectors of the noncanonical cyclic nucleotides cCMP and cUMP is crucial to claim a role as true second messenger for these molecules. In a complementary approach with classic biochemistry and pharmacology techniques, the DMR provided further evidence that cCMP and cUMP exert effects via the activation of the protein kinases A and G. Additionally, a further target could be uncovered that is distinct from Epac (Desch et al. 2010; Wolter et al. 2011, 2014, 2015; Beckert et al. 2014). A potential involvement of HCN channels in the target spectrum needs to be further addressed using unbiased techniques, provided the availability of tool compounds to effectively inhibit HCN activity. A distinct inactivation pattern of cCMP and cUMP provokes the question to which extent PDEs control the kinetic of the cell response. Selective inhibition of specific PDE isoforms and subsequent DMR analysis may shed light into these questions.

Despite the certain degree of signaling promiscuity, an important finding of the investigations using DMR technology was the confirmation of distinct signaling phenotypes of cCMP and cUMP just as the discrimination of cCMP to cGMP and cAMP in vivo effects was decisive in the process of appreciating noncanonical cyclic nucleotides as bona fide second messenger (Ervens et al. 1991).

Hampered research progress might arise from perspectives that are locked up in unchallenged patterns of thoughts. Equally ineffective is a lack of direction, seeking starting points for further exploration. Unbiased methods, such as DMR, can expand the view and thereby help to overcome both of these obstacles – either by allowing a glimpse into the cellular processes beyond beaten paths or by providing indications and starting points for further investigation.

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The Chemistry of the Noncanonical Cyclic Dinucleotide 2′3′-cGAMP and Its Analogs

Frank Schwede, Hans-Gottfried Genieser, and Andreas Rentsch

Contents

Abstract

The cyclic dinucleotides (CDNs) cyclic diguanosine monophosphate (c-diGMP) and cyclic diadenosine monophosphate (c-diAMP) with two canonical 3′→5′ internucleotide linkages are ubiquitous second messenger molecules in bacteria, regulating a multitude of physiological processes. Recently the noncanonical CDN cyclic guanosine monophosphate-adenosine monophosphate (2′3′-cGAMP) featuring a mixed linkage, which consists of a 2′→5′ and a 3′→5′ internucleotide bond, has been identified as a signaling molecule in metazoan species in late 2012. 2′3′-cGAMP formation is biocatalyzed by cGAMP synthase (cGAS) upon sensing of cytosolic double-stranded DNA (dsDNA) and functions as an endogenous inducer of innate immunity by directly binding to and activating the adaptor protein stimulator of interferon genes (STING). Thereby 2′3′-cGAMP can stimulate interferon-β (INF-β) secretion, a major signaling pathway of host defense, which is independent of toll-like receptor (TLR) activation. Medicinal chemistry of 2′3′-cGAMP and development of

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corresponding analogs are still in their infancy, and only a handful of structurally related compounds are available to the scientific community. The aim of this chapter is to summarize synthetic approaches to prepare canonical and noncanonical endogenous CDNs including 2′3′-cGAMP. Furthermore, we will describe syntheses of 2′3′-cGAMP analogs bearing modifications, which will facilitate further studies of the emerging biological functions of 2′3′-cGAMP and to identify additional receptor proteins. Finally, we will review latest developments concerning 2′3′-cGAMP analogs with improved hydrolytic stability in cell cultures and in tissues, putatively qualifying for new therapeutic options on the basis of 2′3′-cGAMP signaling.

Keywords
2′3′-cGAMP • c-diGMP • CDN • cGAMP • cGAS • Chemical cGAMP analogs • Rp,Rp-2′3′-c-diAMPSS • STING

1 Introduction

Cyclic diguanosine monophosphate, also known as cyclic bis(3′→5′) diguanylic acid (c-diGMP; c[G(3′,5′)pG(3′,5′)p]), was described in 1987 as an unusual cyclic dinucleotide and allosteric modulator of bacterial cellulose synthase in *Acetobacter xylinum* (Ross et al. 1987). c-diGMP consists of two guanosine-5′-monophosphates (5′-GMP) forming a 12-membered ring system that contains two phosphate diester units with canonical 3′,5′-bonding (Fig. 1). Meanwhile, c-diGMP is a well-established universal second messenger in the bacterial kingdom with multiple functions, including biofilm formation, motility, and virulence (Jenal and Malone 2006; Römling et al. 2013).

Cyclic diadenosine monophosphate (c-diAMP; c[A(3′,5′)pA(3′,5′)p]; Fig. 1) was identified in 2008 as a signaling nucleotide involved in the regulation of DNA integrity in *Bacillus subtilis* during sporulation (Witte et al. 2008). In the following years, c-diAMP was found in multiple other bacteria and is now considered as an emerging bacterial second messenger with a steadily increasing number of reports unraveling new cellular functions in prokaryotes (Corrigan and Gründling 2013; Commichau et al. 2015).

Cyclic guanosine monophosphate-adenosine monophosphate (cyclic GMP-AMP; cGAMP) with a combination of adenine and guanine nucleobases was published as the first endogenous CDN representing a second messenger function in metazoa only very recently in 2013 (Sun et al. 2013; Wu et al. 2013). cGAMP is biosynthesized from ATP and GTP by cGAMP synthase (cGAS) upon sensing of cytosolic dsDNA, a typical danger signal in eukaryotic cells. Subsequently, cGAMP activates the adaptor protein stimulator of interferon genes (STING) to induce type 1 interferons and other cytokines as response of the innate immune system. Metazoan cGAMP was originally described as CDN with two canonical 3′,5′-linkages between the 5′-GMP and 5′-AMP units, similar to the

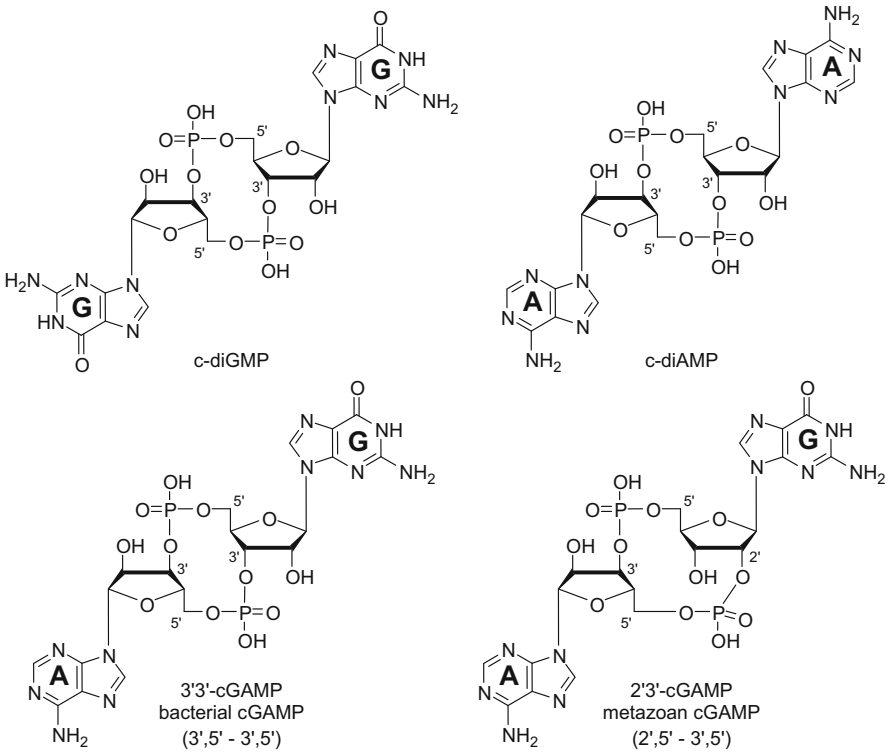


Fig. 1 Canonical and noncanonical natural cyclic dinucleotides (CDNs). All structural drawings in this chapter depict the free acid form of the phosphate moieties in the 12-membered ring system. At physiological pH each of the phosphate diesters is negatively charged

almost simultaneously identified bacterial 3′3′-cGAMP (c[G(3′,5′)pA(3′,5′)p]) (Fig. 1) from *Vibrio cholerae* (Davies et al. 2012). Within some months, several labs investigated the mode of action of metazoan cGAS and characterized the mixed CDN formed with multiple methods, such as crystallography, enzymatic hydrolysis, and chemical synthesis of cGAMP isomers with defined chemical structures (Ablasser et al. 2013; Civril et al. 2013; Diner et al. 2013; Gao et al. 2013; Kranzusch et al. 2013; Zhang et al. 2013). Thereby it was confirmed that in metazoan cGAMP the internucleotide phosphate-binding motifs constituted of a quite uncommon combination of a noncanonical 2′,5′-linkage together with a canonical 3′,5′-linkage (2′3′-cGAMP; c[G(2′,5′)pA(3′,5′)p]; Figs. 1 and 2). The phosphodiester-containing 12-membered ring system in 2′3′-cGAMP is at least theoretically rather flexible and can adopt the *syn*- or *anti*-conformation with respect to the two nucleobases adenine and guanine. Also the orientation of each nucleobase toward its dedicated ribose can exist in *syn*- and *anti*-conformation as a result of free rotation around the glycosidic bond (N9-C1′). From all hypothetical conformers, in crystals of 2′3′-cGAMP bound to the homodimeric STING protein,

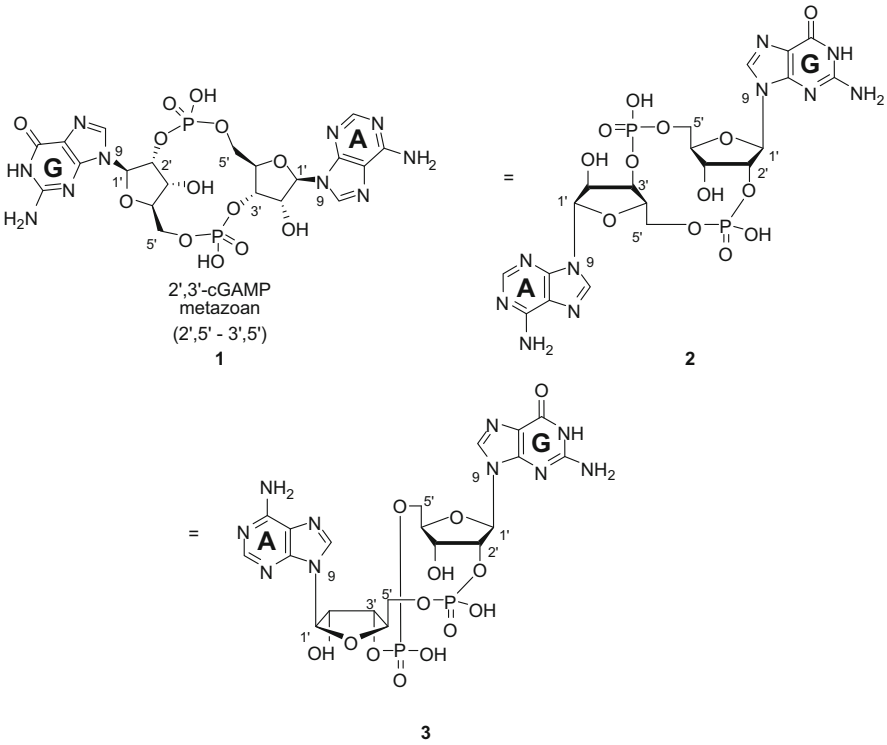


Fig. 2 Various structural presentations (**1**, **2**, **3**) of metazoan 2′3′-cGAMP. All structures are presented in the *anti*-conformation between nucleobase and corresponding ribose. Structure **3** visualizes in particular the *syn*-conformation of the nucleobases adenine and guanine toward each other. In 2′3′-cGAMP-STING crystals, this *syn*-conformation of nucleobase-nucleobase orientation is found together with the *anti*-conformation of both nucleobase-ribose units (Gao et al. 2013; Zhang et al. 2013)

the nucleobases adenine and guanine are oriented *syn* to each other and *anti* to their corresponding ribose unit (Gao et al. 2013; Zhang et al. 2013). Structure **3** in Fig. 2 depicts these geometric characteristics of STING-bound 2′3′-cGAMP with higher accuracy compared to structures **1** and **2**.

There is dramatically increasing evidence that metazoan 2′3′-cGAMP with its immunomodulatory activity can serve as lead for a novel therapeutic paradigm as adjuvant for vaccines or to combat cancer, HIV, and other major diseases related to pathogenic viruses, bacteria, and protozoa. The biological properties of 2′3′-cGAMP are reviewed in detail in Mankan et al. (2017) of this book series, whereas the scope of this chapter is to provide a short overview on synthetic routes for the design of canonical and noncanonical CDNs, like 2′3′-cGAMP and related structures. Furthermore, we will describe syntheses which introduce chemical modifications into noncanonical CDNs. The resulting analogs, featuring modulated biological activities, are versatile chemical tools to further investigate the cGAS-

2′3′-cGAMP-STING pathway and beyond. Lastly, we will summarize very recent efforts of preclinical testing with 2′3′-cGAMP analogs in cancer pharmacology that may translate into early clinical development in the near future.

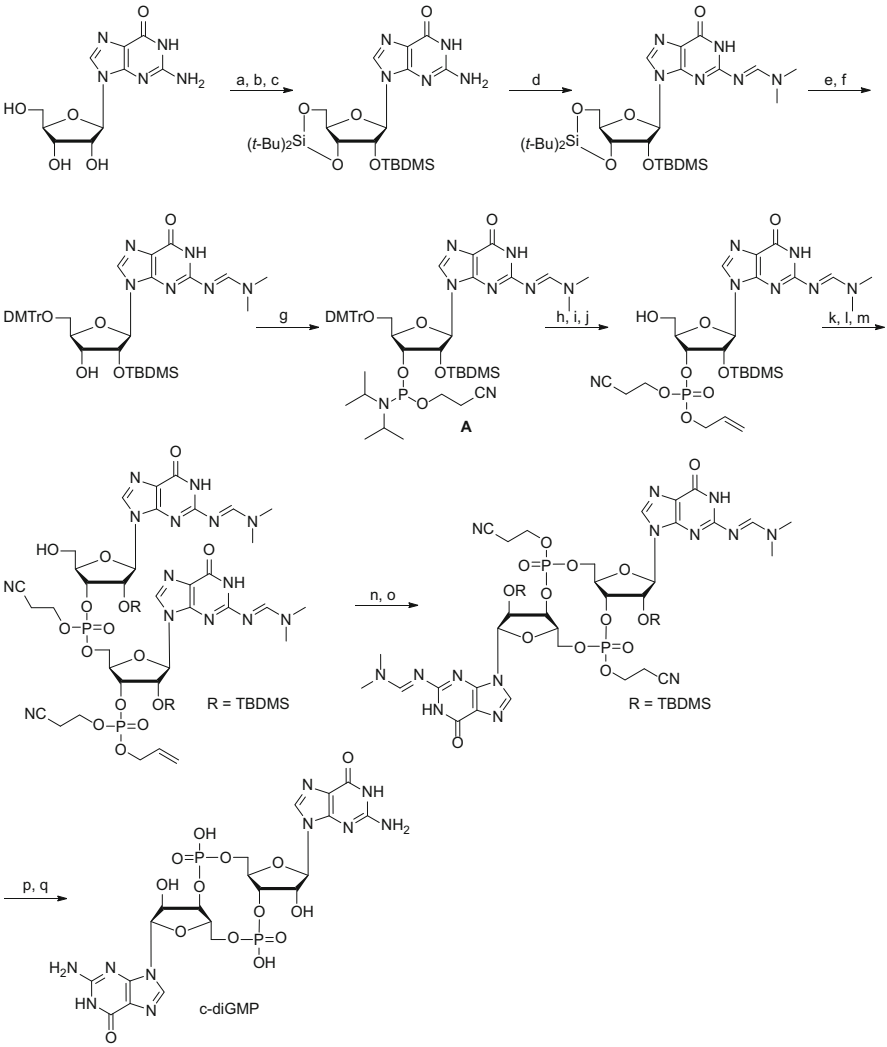
2 Syntheses of Canonical CDNs and 2′3′-cGAMP

Over the last 30 years, several syntheses of c-diGMP and c-diAMP have been published, applying phosphate, phosphonate, and phosphite chemistries in solution phase. The majority of these synthetic protocols rely on nucleoside precursors with suitable protection groups to allow for regiospecific reaction steps (Hsu and Dennis 1982; Ross et al. 1990; Hayakawa et al. 2003; Hyodo and Hayakawa 2004; Zhang et al. 2004; Yan and Aguilar 2007; Zhao et al. 2009; Ching et al. 2010; Gaffney et al. 2010). A typical synthetic setup is exemplified in Scheme 1 (Hyodo and Hayakawa 2004).

Several of these protocols were also applied for the synthesis of CDNs bearing two heterogenic nucleobases, like guanine and adenine for the generation of 3′3′-cGAMP, and are carefully reviewed by Shanahan and Strobel (2012). In general, this "nucleoside approach" is convenient for the preparation of small collections of CDNs differing in their nucleobase composition. However, it should be emphasized that most of these protocols include more than 10 synthetic steps accompanied by several chromatographic purifications. In the final CDN the nucleoside combination is defined by the initial selection of nucleoside precursors. This implies that each synthetic process is only suitable to prepare a single cyclic dinucleotide with a unique nucleobase signature. To this end, all these synthetic procedures are not compatible with combinatorial chemistry strategies, a severe drawback limiting the access to a large number of CDN analogs. This obstacle was tackled at least in part by Giese and coworkers (Amiot et al. 2006) with the preparation of a common cyclic ribose-3′,5′-3′,5′-diphosphate building block that was fused with a nucleobase only at the end of the multistep reaction (Scheme 2).

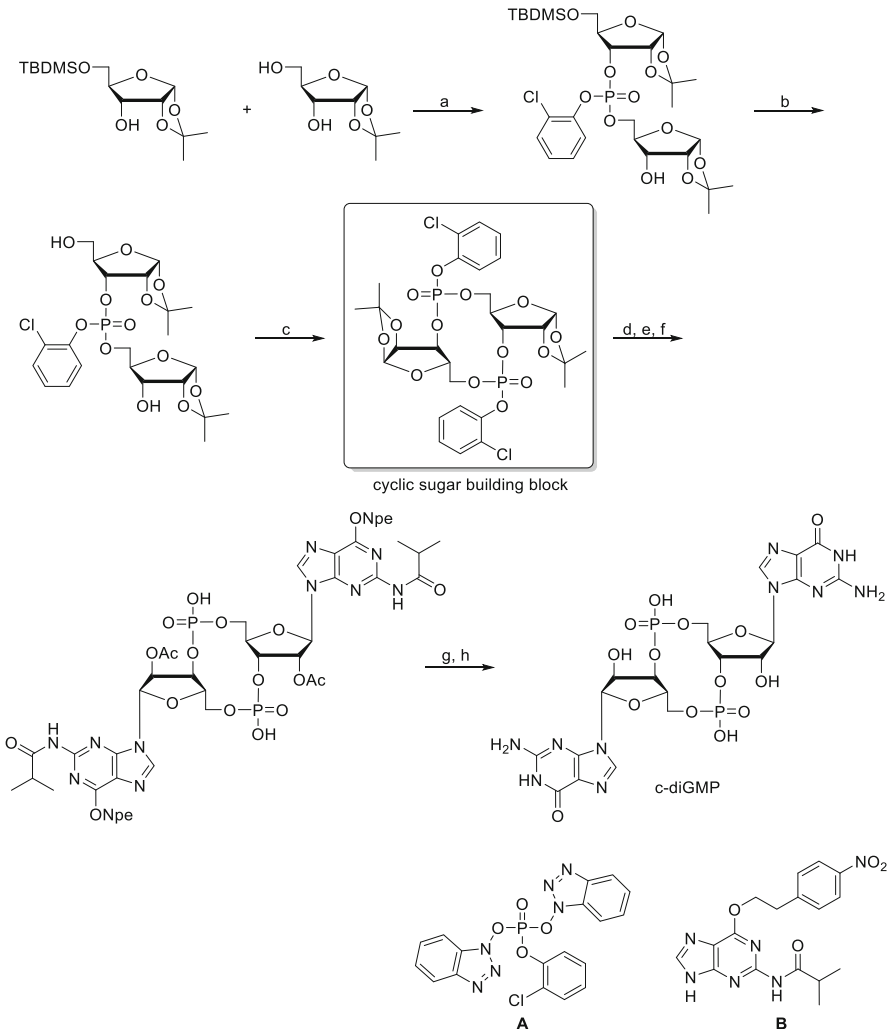
As it stands for now, this improved flexibility of the "backbone approach" was reported only for the preparation of CDNs with identical nucleobases, including c-diGMP and c-diAMP. The generation of heteromeric CDNs should be possible by employing a 1:1 mixture of two distinct nucleobases in the final ribosylation step. However, subsequent separation and purification of heteromeric CDNs and homomeric by-products by HPLC are expected to be challenging and might be prohibitive for a scale-up of this method. In the future, a further elaborated "backbone approach" might qualify for combinatorial strategies to fuse a large number of preactivated nucleobases with a universal cyclic backbone intermediate for the generation of CDN libraries.

While all these reactions are based on solution chemistry, Sintim and coworkers reported a solid-phase approach for the preparation of c-diGMP using a DNA synthesizer and two different guanosine phosphoramidites, including 5′-DMTr-2′-O-TBDMS-guanosine phosphoramidite, carrying a methyl phosphate protection



Scheme 1 Exemplary "nucleoside approach" protocol for the synthesis of c-diGMP (Hyodo and Hayakawa 2004). Synthetic steps: (*a*) $(t\text{-Bu})_2Si(OTf)_2$, 0°C; (*b*) imidazole; (*c*) TBDMSCl, 60°C; (*d*) $(CH_3)_2NCH(OCH_3)_2$, 50°C; (*e*) HF·pyridine, pyridine, CH_2Cl_2, 0°C; (*f*) DMTrCl, pyridine; (*g*) $NCCH_2CH_2OP[N(i\text{-}C_3H_7)_2]Cl$, 2,4,6-collidine, *N*-methylimidazole, THF; (*h*) allyl alcohol, IMP, 3 Å molecular sieves, MeCN; (*i*) BPO, toluene; (*j*) DCA, CH_2Cl_2; (*k*) *A*, IMP, 3 Å molecular sieves, MeCN; (*l*) BPO, toluene; (*m*) DCA, CH_2Cl_2; (*n*) NaI, acetone, reflux; (*o*) TPSCl, *N*-methylimidazole; (*p*) conc. aqueous NH_3/MeOH (1:1 v/v), 50°C; (*q*) $Et_3N{\cdot}3HF$

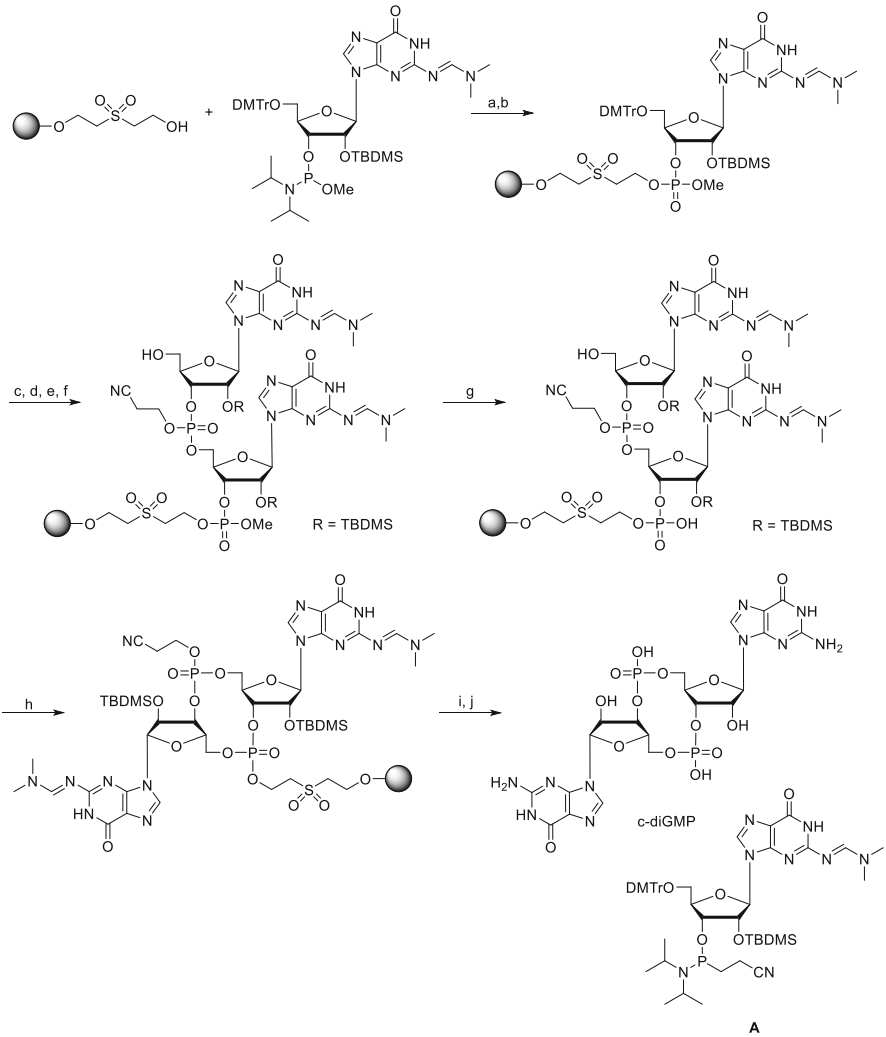
group (Scheme 3) (Kiburu et al. 2008). In this protocol, the cyclization step with 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) in pyridine (step h in Scheme 3) to prepare the protected CDN was critical with only moderate yields below 50%. Subsequently, in a second-generation approach, the authors cleaved off



Scheme 2 "Backbone approach" with common cyclic sugar building block for the synthesis of c-diGMP and other canonical CDNs (Amiot et al. 2006). Synthetic steps: (*a*) *A*, THF, 4 Å molecular sieves; (*b*) CAN, MeOH; (*c*) 2-chlorophenyl phosphorodichloridate, pyridine; (*d*) AcOH, Ac_2O, H_2SO_4; (*e*) *B*, *N*,*O*-Bis(trimethylsilyl)acetamide (BSA), $ClCH_2CH_2Cl$ (DCE), 80°C; (*f*) TMSOTf, toluene, 80°C; (*g*) *syn*-pyridine-2-carbaldoxime, *N*,*N*,*N*,*N*-tetramethylguanidine; (*h*) aqueous NH_3, 50°C

the protected linear dimer from the solid support prior to quantitative cyclization with MSNT in solution (Kiburu et al. 2008).

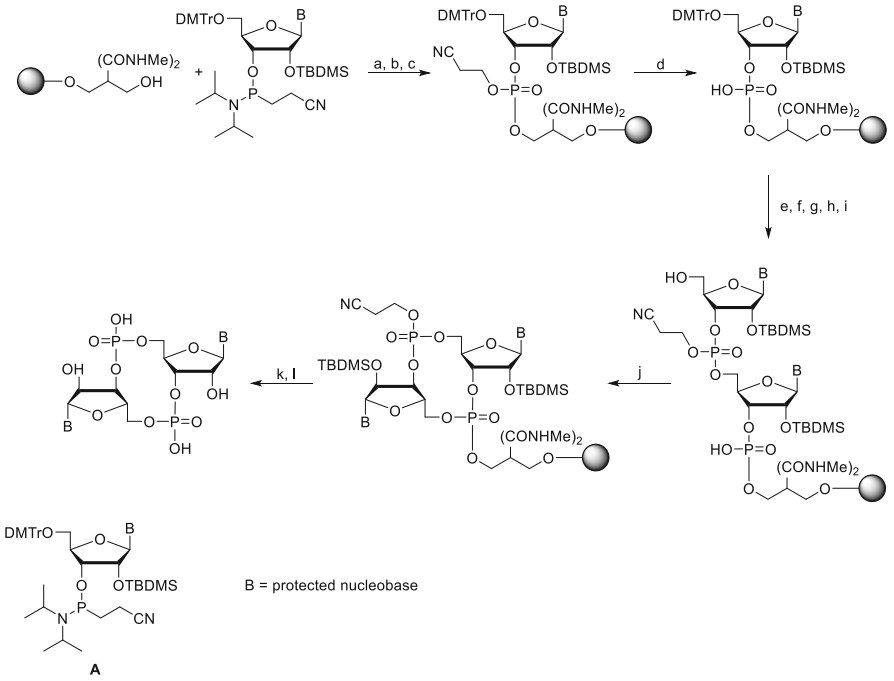
Strobel and coworkers further developed syntheses in heterogenic phase (Scheme 4) making use of the most common and commercially available nucleoside phosphoramidites with 5′-DMTr, 2′-O-TBDMS protecting groups, as well as



Scheme 3 Solid-phase synthesis of c-diGMP with two distinct nucleoside phosphoramidite precursors (Kiburu et al. 2008). Synthetic steps: (*a*) 1*H*-tetrazole, MeCN; (*b*) I_2, pyridine, H_2O; (*c*) DCA, CH_2Cl_2; (*d*) *A*, 1*H*-tetrazole, MeCN; (*e*) I_2, pyridine, H_2O; (*f*) DCA, CH_2Cl_2; (*g*) S_2Na_2; (*h*) MSNT (0.1 M), pyridine, 48 h; (*i*) aqueous NH_3; (*j*) $Et_3N{\cdot}3HF$

3′-O-[(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphinyl] functionalization (Smith et al. 2010; Shanahan et al. 2011; Smith et al. 2011; Luo et al. 2012; Shanahan et al. 2013).

Quantitative cyclization to generate the protected CDN was accomplished on bead by repeated prolonged treatment with MSNT in pyridine combined with intermittent pyridine washing steps (step j in Scheme 4). As mostly automated syntheses, these solid support methods could be advantageous to solution chemistry

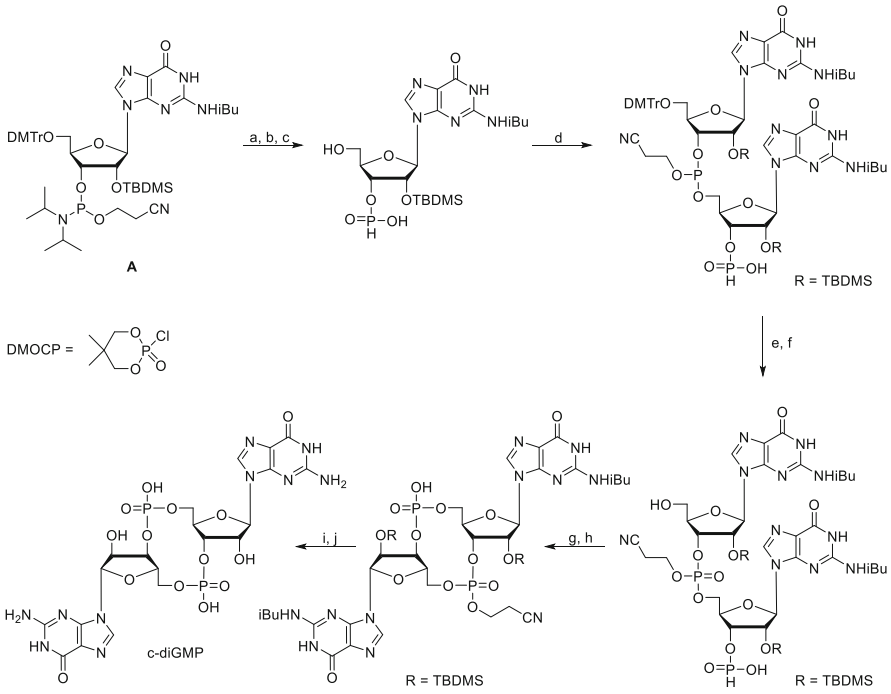


Scheme 4 Solid-phase synthesis of canonical CDNs with common 5′-DMTr-2′-O-TBDMS-3′-O-[(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphinyl]nucleoside precursors (Shanahan et al. 2011). Synthetic steps: (*a*) 1*H*-tetrazole, MeCN; (*b*) *t*-BuOOH; (*c*) Ac_2O, NH_2Me; (*d*) TEA/MeCN; (*e*) DCA, CH_2Cl_2; (*f*) *A*, 1*H*-tetrazole, MeCN; (*g*) *t*-BuOOH; (*h*) Ac_2O, NH_2Me; (*i*) TEA/MeCN; (*j*) MSNT (0.1 M), pyridine, 72–96 h; (*k*) aqueous NH_3; (*l*) $Et_3N{\cdot}3HF$

to prepare multiple CDNs in a shorter time frame. They could therefore represent a good starting point for effective scale-up and process development of CDN synthesis in the future.

Large-scale and cost-saving syntheses of CDNs are eagerly awaited by the scientific community to have at command sufficient quantities of these second messengers for detailed biological and pharmacological testing. To meet these demands Jones and coworkers developed a solution-phase method, which gives access to c-diGMP as well as related phosphorothioate analogs in a gram scale and is currently considered as state of the art (Scheme 5) (Gaffney et al. 2010).

It uses the common and commercially available precursor **A** (N^2-iBu-5′-O-DMTr-2′-O-TBDMS-3′-O-CEP-guanosine, compare Scheme 5) and is based on phosphoramidite/phosphonate-chemistry protocols established before by this laboratory (Zhang et al. 2004; Zhao et al. 2009). The innovation, however, lies in a carefully chosen synthetic route, applying reaction conditions that allow for the whole process to be performed in a single-flask synthesis without the need for any intermediate purification steps. This improved protocol thus demonstrates a very straightforward and scalable method. Furthermore, after the final deprotection,



Scheme 5 Synthesis of c-diGMP without chromatographic purification steps (Gaffney et al. 2010). Synthetic steps: (*a*) pyr·TFA/H_2O; (*b*) *t*-$BuNH_2$; (*c*) DCA/H_2O; (*d*) *A*, pyridine; (*e*) *t*-BuOOH; (*f*) DCA/H_2O; (*g*) DMOCP; (*h*) I_2, H_2O; (*i*) *t*-$BuNH_2$; (*j*) $Et_3N{\cdot}3HF$

c-diGMP is simply crystallized from the reaction mixture, avoiding cost-intensive chromatographic purifications throughout the whole reaction sequence. Also the cyclic diguanosine monophosphorodithioate isomers Rp,Rp-c-diGMPSS (c[G(3′,5′)pS-G(3′,5′)pS:Rp,Rp]) and Rp,Sp-c-diGMPSS (c[G(3′,5′)pS-G(3′,5′)pS:Rp,Sp]) were prepared with this improved method employing sulfurization instead of oxidation reagents (Scheme 5, Fig. 3).

Here, only a single purification step for the separation of Rp,Rp- and Rp,Sp-isomers is necessary. In these dithiophosphates of c-diGMP, the terms “Rp” and “Sp” refer to the different isomers of the sulfur-modified and thus chiral phosphorus atoms of the 12-membered ring system of cyclic dinucleotides according to the Cahn-Ingold-Prelog priority rules (CIP). The isomer Sp,Sp-c-diGMPSS (c[G(3′,5′)pS-G(3′,5′)pS:Sp,Sp]; (Zhao et al. 2009) is chemically not accessible by this synthetic protocol as a result of the stereoselective introduction of sulfur into the phosphonate intermediate with 3-H-1,2-benzodithiol-3-one (compare Fig. 3, step h; Scheme 5).

Shortly after the first reports of metazoan cGAMP (Sun et al. 2013; Wu et al. 2013), Jones and coworkers adapted their single-flask c-diGMP protocol to be applicable for the synthesis of mixed cyclic dinucleotides containing adenine

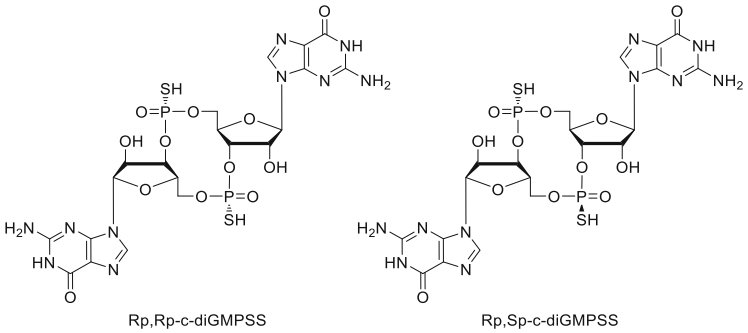
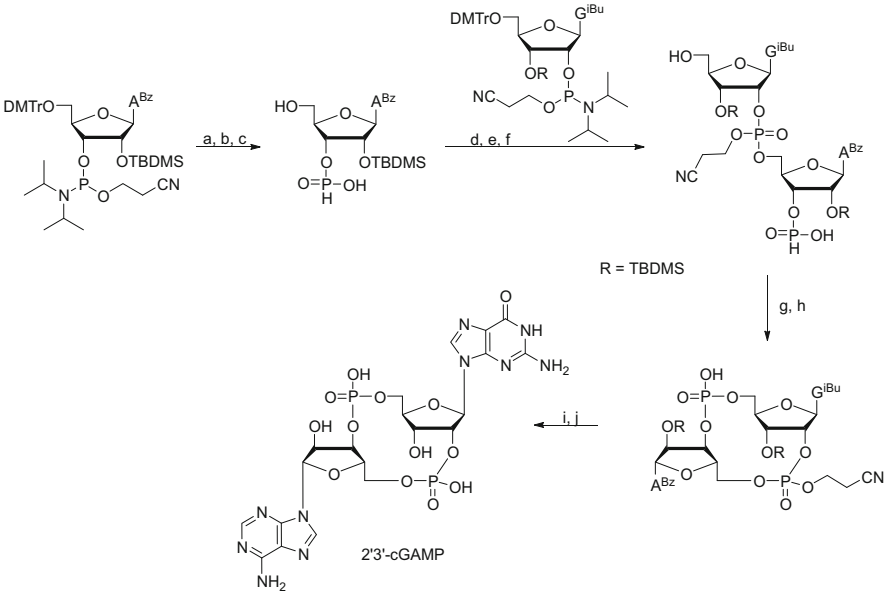


Fig. 3 Chemical structures of Rp,Rp-c-diGMPSS and Rp,Sp-c-diGMPSS (Gaffney et al. 2010). Synthetic steps (compare Scheme 5): (*a*) pyr·TFA/H_2O; (*b*) *t*-BuNH_2; (*c*) DCA/H_2O; (*d*) *A*, pyridine; (*e*) 3-((*N*,*N*-dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione (DDTT); (*f*) DCA/H_2O; (*g*) DMOCP; (*h*) 3-H-1,2-benzodithiol-3-one, silica gel chromatographic separation of isomers; (*i*) *t*-BuNH_2; (*j*) $Et_3N{\cdot}3HF$



Scheme 6 Preparation of 2′3′-cGAMP (Gao et al. 2013). Synthetic steps: (*a*) pyr·TFA/H_2O; (*b*) *t*-BuNH_2; (*c*) DCA/H_2O; (*d*) pyridine; (*e*) *t*-BuOOH; (*f*) DCA/H_2O; (*g*) DMOCP; (*h*) I_2, H_2O; (*i*) $MeNH_2$; (*j*) $Et_3N{\cdot}3HF$

and guanine (Gao et al. 2013). With the modified procedure in hands, they succeeded in preparing several cGAMP isomers with canonical and noncanonical linkages. In particular, to facilitate the 2′-5′ linkages, they interchanged the 2′- and 3′-functionalities of the second precursor (Scheme 6).

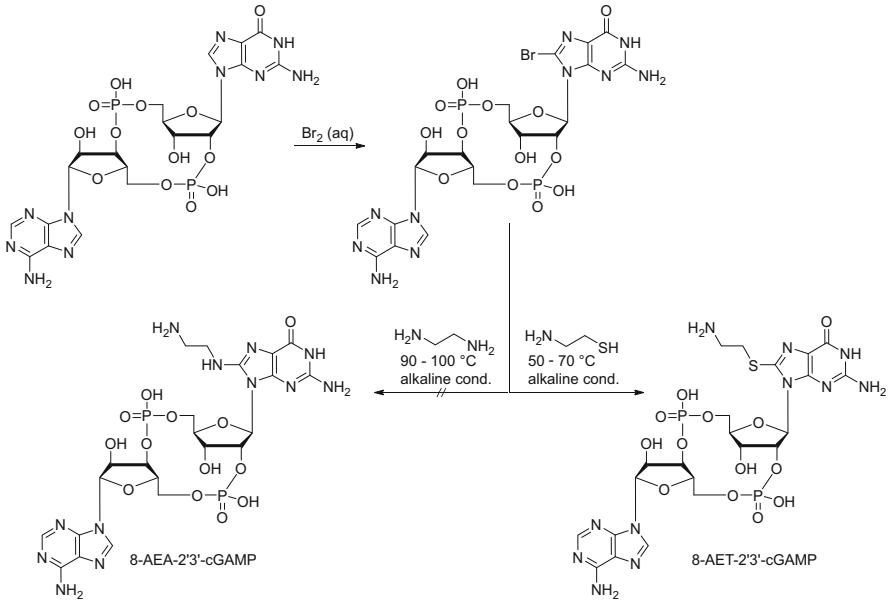
The synthetically prepared and structurally defined 2′3′-cGAMP, applied as HPLC reference, helped to confirm the chemical identity of native metazoan cGAMP. Meanwhile, this single-flask protocol was successfully established in other laboratories, partially modified and used to prepare 2′3′-cGAMP and analogs in larger scale (D. Kashin et al., unpublished results; Dubensky et al. 2014; Zhang et al. 2013)

3 Syntheses of 2′3′-cGAMP Analogs

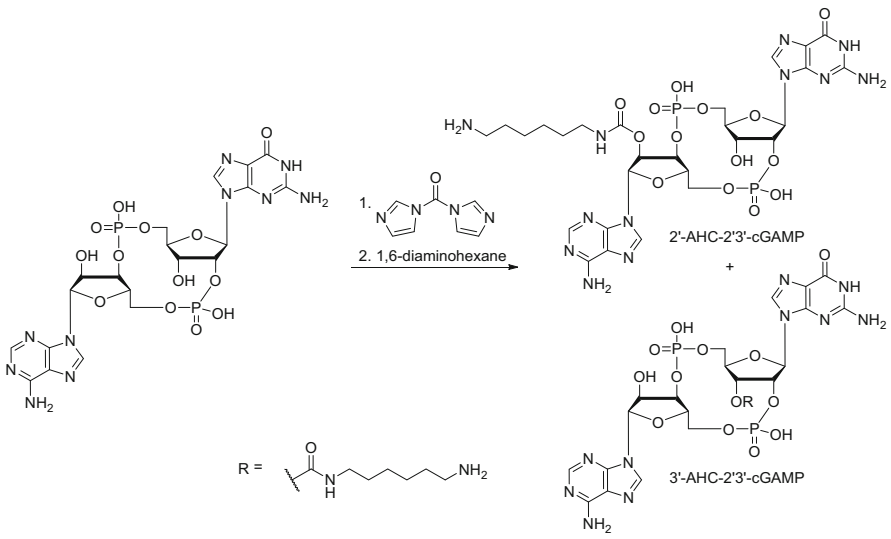
3.1 Analogs with Modifications in the Nucleobase and Ribose Moieties

2′3′-cGAMP analogs with meaningful chemical modifications are thought to be useful tools for a more detailed insight into the recently unraveled cGAS-2′3′-cGAMP-STING pathway. They can also support the identification of additional receptor proteins in this emerging scientific field of noncanonical cyclic dinucleotides in eukaryotes. We set out to generate 2′3′-cGAMP analogs with functional spacers in different positions of the molecule to be employed for interaction studies with STING and for a chemical proteomics approach to search for additional binding proteins. For this task, we selected position 8 of the guanine moiety and the 2′-OH/3′-OH groups of the ribose parts of 2′3′-cGAMP for a first generation of chemical interventions. Regioselective introduction of bromine into position 8 of guanine was accomplished by reacting 2′3′-cGAMP with bromine in aqueous solution at ambient temperature (Scheme 7). Under these conditions only guanine, but not the equivalent position in adenine, is susceptible to bromination, leading to 8-Br-2′3′-cGAMP (c[8-Br-G(2′,5′)pA(3′,5′)p]), the first 2′3′-cGAMP analog with a single modification in the nucleobase.

Nucleophilic substitutions of 8-bromoguanine-containing nucleosides and nucleotides with standard aminoalkylamino reagents are only possible in sealed containers under harsh conditions at temperatures of up to 160°C (Ikehara and Muneyama 1966; Brown et al. 1993). The alternative thiol groups in ω-aminoalkylthiol reagents are better nucleophiles and commonly used to connect terminal aminoalkyl spacers with thioether linkage to position 8 of guanine. Therefore, 8-Br-2′3′-cGAMP was further reacted with aminoethylthiol (AET) under alkaline conditions at moderate temperatures (50-70°C) to yield cyclic 8-AET-2′3′-cGAMP (c[8-AET-G(2′,5′)pA(3′,5′)p]) (Scheme 7). Introduction of hexyl spacers with a terminal amino group into 2′- and 3′-positions of 2′3′-cGAMP was accomplished according to Hammerschmidt et al. by initial activation of the lipophilic bis-diisopropylethylammonium salt of 2′3′-cGAMP with 1,1′-carbonyl-diimidazole in anhydrous DMF, followed by addition of excess 1,6-diaminohexane (Hammerschmidt et al. 2012). The raw mixture of 2′-AHC-2′3′-cGAMP (c[G(2′,5′)p-2′-AHC-A(3′,5′)p]) and 3′-AHC-2′3′-cGAMP (c[3′-AHC-G(2′,5′)pA(3′,5′)p]) (Scheme 8) was purified by HPLC to isolate the spacer-modified 2′- and 3′-isomers of 2′3′-cGAMP. All analogs with functionalized spacers can serve as precursors for



Scheme 7 Regioselective syntheses in position 8 of guanine to generate 8-Br-2′3′-cGAMP and ω-aminoethylthio spacer-containing 8-AET-2′3′-cGAMP



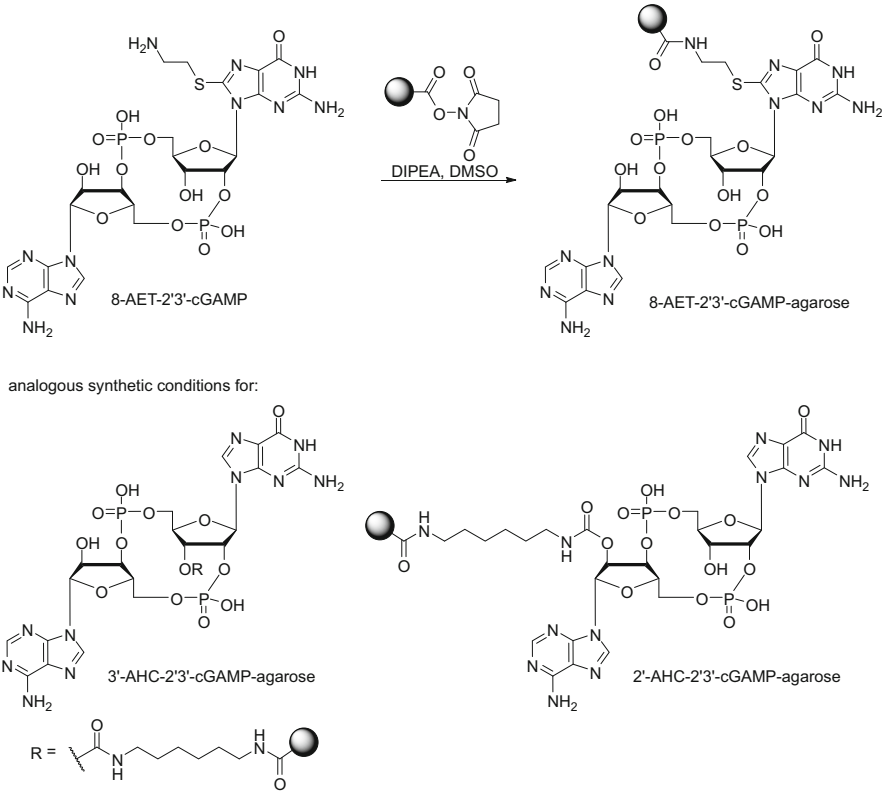
Scheme 8 Modifications of 2′3′-cGAMP in the 2′- and 3′-ribose position with a reactive ω-aminohexyl spacer group

further chemical modifications, including immobilization on agarose beads, coupling to chips for surface plasmon resonance experiments, and labeling with reporter groups like biotin or fluorescent dyes.

Furthermore, after coupling to haptens these functionalized 2′3′-cGAMP analogs can be suitable tools for antibody generation and subsequent development of immunoassays for 2′3′-cGAMP. Such immunoassays could be employed to simplify the detection and quantification of 2′3′-cGAMP in biological matrices. Currently, the state of the art is still highly sophisticated HPLC-MS/MS analytics, which has sufficient detection sensitivities, but depends on high-end HPLC-MS hardware, precluding its broad application as routine method (compare Bähre and Kaever (2017) of this book series).

Immobilization on agarose beads was initiated by reacting the spacer-containing 2′3′-cGAMP analogs with *N*-hydroxysuccinimide ester (NHS)-activated agarose in anhydrous DMSO in the presence of a non-nucleophilic base like diisopropylethylamine as described in Bertinetti et al. (Scheme 9) (Bertinetti et al. 2009).

Potential applications of the resulting tools 8-AET-2′3′-cGAMP-agarose, 2′-AHC-2′3′-cGAMP-agarose, and 3′-AHC-2′3′-cGAMP-agarose are not only affinity



Scheme 9 Syntheses of various 2′3′-cGAMP-agaroses

purifications of STING, but also the screening for additional interaction partners of STING, which should co-elute during chromatography or co-precipitate in pulldown experiments. Furthermore, these agaroses could be employed in the quest for new binding proteins of 2′3′-cGAMP in metazoa.

In this regard, it is of interest that CDNs, including 2′3′-cGAMP, have been reported to bind to a newly identified C-linker binding pocket (CLP) in the cytosolic C-linker region of the hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4), the main isoform of the cardiac pacemaker (Lolicato et al. 2014). HCN4 is activated by hyperpolarization of membrane voltage in myocytes, and its open probability is controlled and prolonged by cAMP binding to the cyclic nucleotide-binding domain, which is separate from the CLP site. Upon binding to CLP, 2′3′-cGAMP and bacterial CDNs were shown to antagonize cAMP regulation of the channel leading to an efficient reduction of heart rate by 30%. This first report about a putative signaling role of 2′3′-cGAMP apart from the innate immunity system highlights that physiological roles of 2′3′-cGAMP in mammals are far from being fully evaluated, including the existence of additional protein-binding partners that may be identified by the chemical proteomics approach with 2′3′-cGAMP-agaroses as affinity baits.

Biotinylated analogs of the canonical bacterial second messengers c-diGMP and c-diAMP were synthesized by Beaucage and coworkers (Grajkowski et al. 2010) and in our laboratory. Biotinylated c-diGMP analogs were shown to interact with certain previously established c-diGMP binders (Luo et al. 2012) and, after coupling to streptavidin beads, were suitable to isolate putative c-diGMP interacting proteins from murine macrophages after challenge with *Legionella pneumophila* (Abdul-Sater et al. 2012). 2′-[Biotin]-AHC-c-diAMP was used to develop a c-diAMP ELISA (Bai et al. 2013) and after immobilization on streptavidin-coated magnetic beads, employed for the identification of new receptor proteins from *Staphylococcus aureus* and *Bacillus subtilis* in pulldown assays (Corrigan et al. 2013; Gundlach et al. 2015). To enable comparable studies in the field of metazoan 2′3′-cGAMP, the ω-aminospacer-containing analogs 8-AET-2′3′-cGAMP, 2′-AHC-2′3′-cGAMP and 3′-AHC-2′3′-cGAMP were reacted with biotinamidocaproate-NHS ester ([Biotin]-NHS) in borate buffer pH 9/2-propanol. Raw mixtures were subsequently purified with ion exchange and reversed phase chromatography to remove unreacted starting material and hydrolyzed biotinamidocaproic acid contaminations, leading to 8-[Biotin]-AET-2′3′-cGAMP (c[8-[Biotin]-AET-G(2′,5′)pA(3′,5′)p]), 2′-[Biotin]-AHC-2′3′-cGAMP (c[G(2′,5′)p-2′-[Biotin]-AHC-A(3′,5′)p]), and 3′-[Biotin]-AHC-2′3′-cGAMP (c[3′-[Biotin]-AHC-G(2′,5′)pA(3′,5′)p]), respectively (Fig. 4).

As was shown for the biotinylated derivatives of bacterial second messengers in recent years, these new biotinylated noncanonical congeners are expected to serve as valuable tools for further characterization of 2′3′-cGAMP signaling in eukaryotes.

The fluorescein-labeled analog 2′-Fluo-AHC-c-diGMP is commercially available and was employed to detect c-diGMP-binding proteins in c-diGMP-agarose

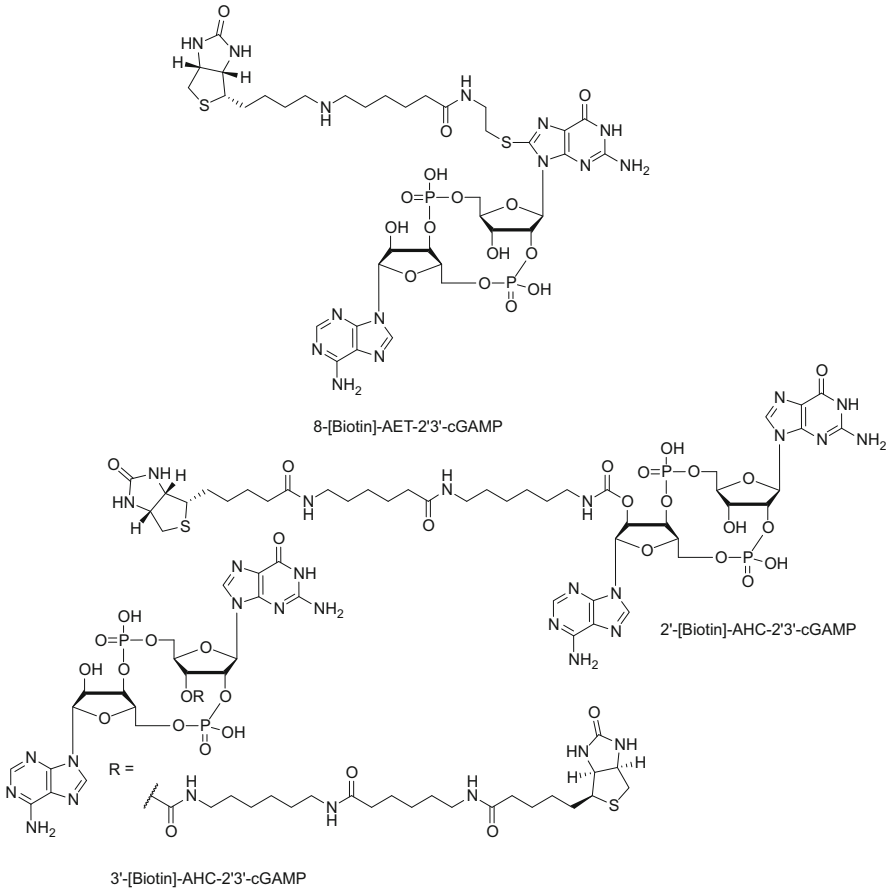


Fig. 4 Chemical structures of biotinylated 2′3′-cGAMP analogs

eluates, thereby avoiding radioactive assays (Düvel et al. 2012). This compound was also used to develop a fluorescence polarization-based assay to measure binding affinities of flavonoid-based agonists of mouse STING that compete for the CDN binding site (Kim et al. 2013). Very recently, 2′-Fluo-AHC-c-diGMP was applied to determine binding affinities of c-diGMP to receptor proteins with microscale thermophoresis coupled to fluorescence detection (Sundriyal et al. 2014). 2′-MANT-c-diGMP and 2′,2″-Di-MANT-c-diGMP containing the fluorescent and environmentally sensitive *N*-methylanthranilic (MANT) dye were used for the characterization of c-diGMP-binding proteins (Sharma et al. 2012) and for staining of putative c-diGMP-binding partners in cytosolic vesicles of macrophages after *Legionella pneumophila* challenge (Abdul-Sater et al. 2012). Given the variety of potential applications for fluorescently labeled 2′3′-cGAMP, we prepared a first series of fluorescein-containing analogs. For this purpose, we

reacted 8-AET-2′3′-cGAMP, 2′-AHC-2′3′-cGAMP, and 3′-AHC-2′3′-cGAMP with 5-carboxyfluorescein-NHS ester in borate buffer pH 9/DMSO. The reaction products 8-Fluo-AET-2′3′-cGAMP (c[8-Fluo-AET-G(2′,5′)pA(3′,5′)p]), 2′-Fluo-AHC-2′3′-cGAMP (c[G(2′,5′)p-2′-Fluo-AHC-A(3′,5′)p]), and 3′-Fluo-AHC-2′3′-cGAMP (c[3′-Fluo-AHC-G(2′,5′)pA(3′,5′)p]) (Fig. 5) were purified by repeated reversed phase chromatography to remove fluorescent impurities. The synthesis of MANT- and di-MANT-substituted 2′3′-cGAMP and other related fluorescent analogs are ongoing projects in our laboratory.

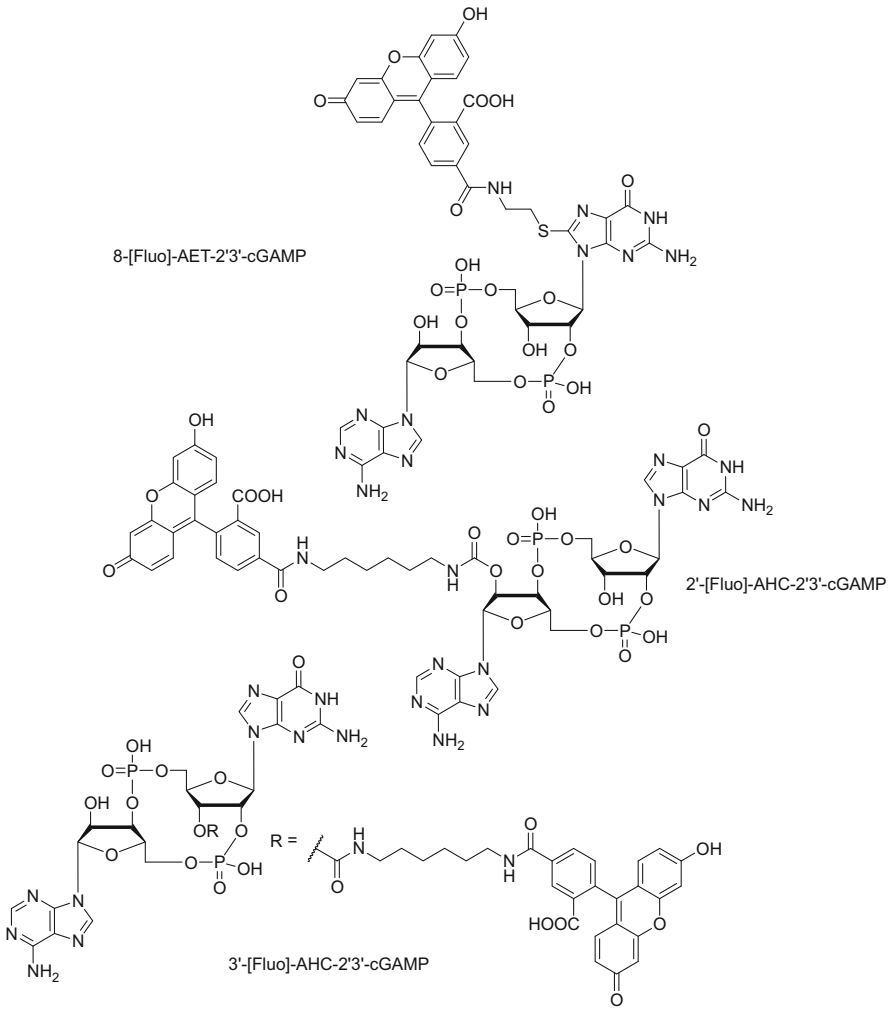
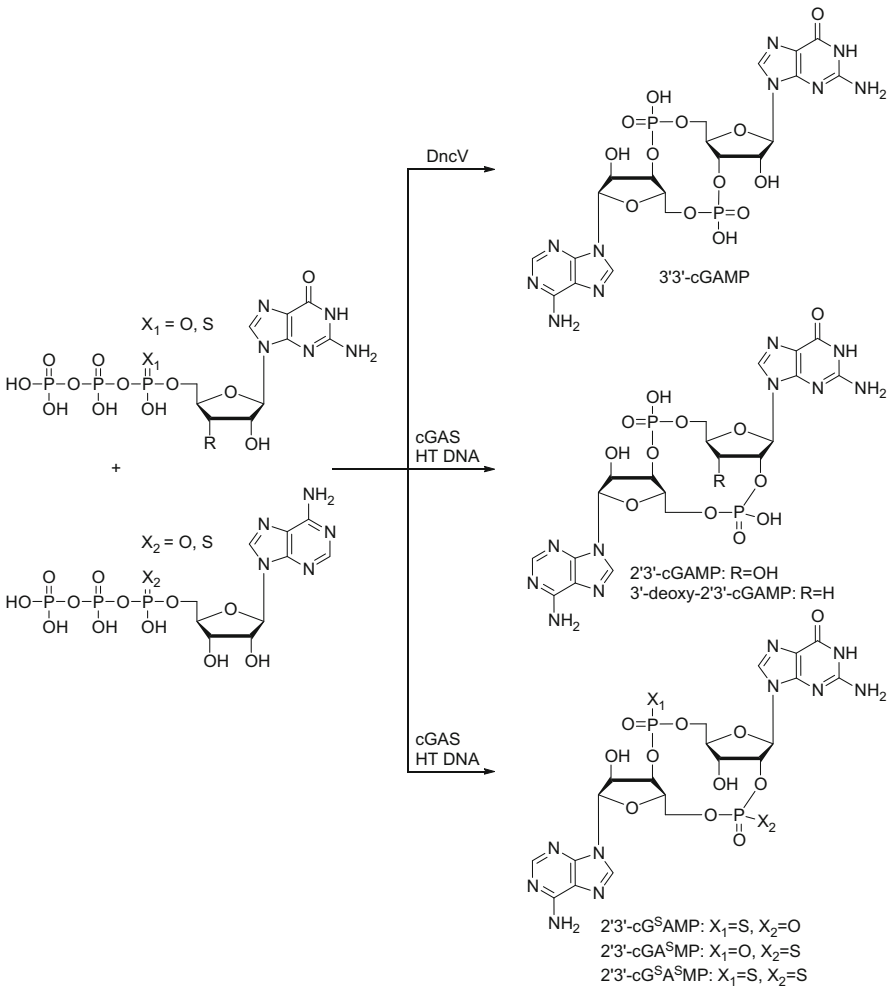


Fig. 5 Chemical structures of fluorescein-labeled 2′3′-cGAMP analogs

4 Syntheses of 2′3′-cGAMP Analogs

4.1 Analogs with Modifications in the Phosphate and Ribose Moieties

In 2014, Mitchison and coworkers identified ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) as dominant endogenous hydrolase of 2′3′-cGAMP in an activity-guided screening and protein purification sequence from calf liver (Li et al. 2014). ENPP1 belongs to the ENPP family consisting of three members (ENPP1-3) and is described as a membrane-associated glycoprotein with an extracellular active site of high ATPase activity. Furthermore, ENPP1 can be released into serum and exists as a functional soluble enzyme (Belli et al. 1993). The protein has only weak substrate specificity and cleaves a variety of pyrophosphate bonds in nucleotides, including ATP, ADP, NAD^+, ADP-ribose, FAD, CoA, and diadenosine polyphosphates, leading to 5′-AMP. Also UDP-glucose and other pyrimidine nucleotides may serve as substrates (Bollen et al. 2000; Cimpean et al. 2004). In incubations with recombinant ENPP1, 2′3′-cGAMP is hydrolyzed efficiently with similar kinetics as the natural substrate ATP. Given this apparent instability of the natural metazoan second messenger, the bacterial 3′3′-cGAMP and a set of 2′3′-cGAMP analogs with modifications in the ribose or the phosphate moiety were synthesized and tested for improved hydrolytic resistance against ENPP1 (Li et al. 2014). 3′3′-cGAMP was prepared chemoenzymatically from ATP and GTP by dinucleotide cyclase (DncV), an endogenous cyclase from *Vibrio cholerae* (Scheme 10) (Davies et al. 2012). All noncanonical CDNs were enzymatically synthesized with cGAS, which was activated by DNA from herring testes (HT DNA), and appropriately modified ATP and GTP analogs. Incubation of 3′-deoxy-GTP with ATP resulted in 3′-deoxy-2′3′-cGAMP (c[3′-dG(2′,5′)pA(3′,5′)p]) (Scheme 10), which showed a half-life of approx. 1 h in ENPP1 hydrolase assays, comparable to 2′3′-cGAMP itself. By contrast, the mono- and dithiophosphate analogs 2′3′-cG^{S}AMP (c[G(2′,5′)pS-A(3′,5′)p]), 2′3′-cGA^{S}MP (c[G(2′,5′)p-A(3′,5′)pS]) with sulfur either in the 2′,5′- or 3′,5′-linkage and 2′3′-cGAMPSS (2′3′-cG^{S}A^{S}MP) (Scheme 10) demonstrated significantly improved hydrolytic stability against ENPP1. The half-life of all thiophosphate-containing CDNs was in the range of 24 h or even longer. This is in accordance with former reports about increased biological stabilities of sulfur-containing nucleoside phosphorothioates compared to unmodified phosphodiesters in cyclic nucleotides, triphosphates, or oligonucleotides (Eckstein and Gish 1989; Schwede et al. 2000; Eckstein 2014). 2′3′-cG^{S}AMP, 2′3′-cGA^{S}MP, and 2′3′-cGAMPSS were prepared by incubation of cGAS with ATP, GTP, ATP-α-S, and GTP-α-S in different combinations. In this particular chemoenzymatic protocol, diastereomeric mixtures of Rp- and Sp-ATP-α-S and GTP-α-S were used for the biocatalyzed syntheses, and the reaction products were not fully characterized with respect to their diastereomeric composition of distinct isomers. A careful evaluation of both potentially stereoselective events, the enzymatic substrate recognition and the cyclization step by cGAS, is thought to be possible with commercially available pure Rp- and



Scheme 10 Chemoenzymatic syntheses of various cGAMP analogs (Li et al. 2014)

Sp-isomers of ATP-α-S and GTP-α-S. 3′-Deoxy-2′3′-cGAMP and 2′3′-cGAMPSS were shown to bind to human STING with high affinities in the low nanomolar concentration range, comparable to endogenous 2′3′-cGAMP. Subsequently, these analogs were further tested against 2′3′-cGAMP for their ability to activate human STING in cell culture in long-term incubations of 24 h. Of all analogs tested, the nonhydrolyzable 2′3′-cGAMPSS exhibited highest levels of INF-β induction in human THP-1 cells expressing STING. The effective concentration (EC_{50}) was ~5 μM for 2′3′-cGAMPSS, approximately tenfold lower compared to 2′3′-cGAMP. From additional experiments with lung fibroblast cells from *Enpp1*$^{-/-}$ mice with similar EC_{50}-values for both CDNs, the authors concluded that the improved EC_{50}-value of 2′3′-cGAMPSS was due to increased biostability compared to 2′3′-cGAMP

(Li et al. 2014). In summary, 2′3′-cGAMPSS was recognized as superior lead for further therapeutic development. Interestingly, also the bacterial 3′3′-cGAMP was found to be stable against hydrolysis by ENPP1. Albeit 3′3′-cGAMP had a 50- to 100-fold reduced binding affinity toward human STING in vitro, its improved hydrolytic stability resulted in similar activatory potentials of 3′3′-cGAMP and 2′3′-cGAMP, with EC_{50}-values of ~50 μM for INF-β induction in human THP-1 cells. These initial results opened up another track for future medicinal chemistry interventions to develop CDN-based STING activators with prolonged stability in biological systems.

Applying the general chemical sequence reported by Gaffney et al. (2010), a series of phosphate-based and phosphorothioate-modified CDNs with canonical and noncanonical linkages have been prepared and tested for their biological activity (Dubensky Jr et al. 2014; Corrales et al. 2015; Fu et al. 2015). Besides phosphate-containing CDNs, also the pure isomers Rp,Rp-2′3′-cGAMPSS, Rp,Sp-2′3′-cGAMPSS, Rp,Rp-2′3′-c-diAMPSS, Rp,Sp-2′3′-c-diAMPSS, Rp,Rp-2′3′-c-diGMPSS, and Rp,Sp-2′3′-c-diGMPSS (Fig. 6) were synthesized from commercially available nucleoside phosphoramidites and purified by HPLC.

The resulting analogs were tested for binding to and activation of STING wild type (WT) and four different haplotypes of human STING protein genotypes (REF, HAQ, AQ, and Q alleles), varying in amino acid positions 71, 230, 232, and 293. These single nucleotide polymorphisms were reported to affect innate immune signaling (Jin et al. 2011; Yi et al. 2013). As reported earlier, highest binding affinity for wild type human $STING^{WT}$ was achieved by analogs with noncanonical linkage, namely 2′3′-cGAMP, Rp,Rp-2′3′-cGAMPSS, and Rp,Rp-2′3′-c-diAMPSS (Corrales et al. 2015). For activity screening each of the five STING variants was stably expressed in HEK293T cells, which are negative in endogenous STING. Induction of INF-β was monitored by co-transfection of a IFN-β-luciferase construct. Bacterial CDNs c-diGMP, c-diAMP, and 3′3′-cGAMP were able to induce IFN-β in genotypes $hSTING^{WT}$, $hSTING^{HAQ}$, and $hSTING^{AQ}$, but not in $hSTING^{REF}$ and $hSTING^{Q}$. By contrast, the noncanonical endogenous 2′3′-cGAMP, Rp,Rp-2′3′-cGAMPSS, Rp,Rp-2′3′-c-diAMPSS, and Rp,Rp-2′3′-c-diGMPSS were able to induce an IFN response in all STING variants tested (Corrales et al. 2015). In an alternative protocol, the murine bone marrow-derived dendritic cell line DC2.4 was used for INF-β induction and to compare noncanonical 2′3′-c-diAMP with its corresponding diphosphorothioate congeners Rp,Rp-2′3′-c-diAMPSS and Rp,Sp-2′3′-c-diAMPSS (Fu et al. 2015). Highest levels of INF-β were detected after treatment with Rp,Rp-2′3′-c-diAMPSS. The isomeric Rp,Sp-2′3′-c-diAMPSS generated only a moderately increased INF-β level, similar to 2′3′-c-diAMP. STING signaling was further evaluated with canonical and noncanonical CDNs in peripheral blood mononuclear cells (PBMC) from human donors with different STING genotypes. In these assays the diphosphorothioated analogs Rp,Rp-2′3′-cGAMPSS, Rp,Rp-2′3′-c-diAMPSS, and Rp,Rp-2′3′-c-diGMPSS induced significantly higher levels of INF-β than canonical or noncanonical CDNs without sulfur modification (Corrales et al. 2015; Fu et al. 2015). As a consequence of their superior and pan-genetic activity, Rp,Rp-isomers of

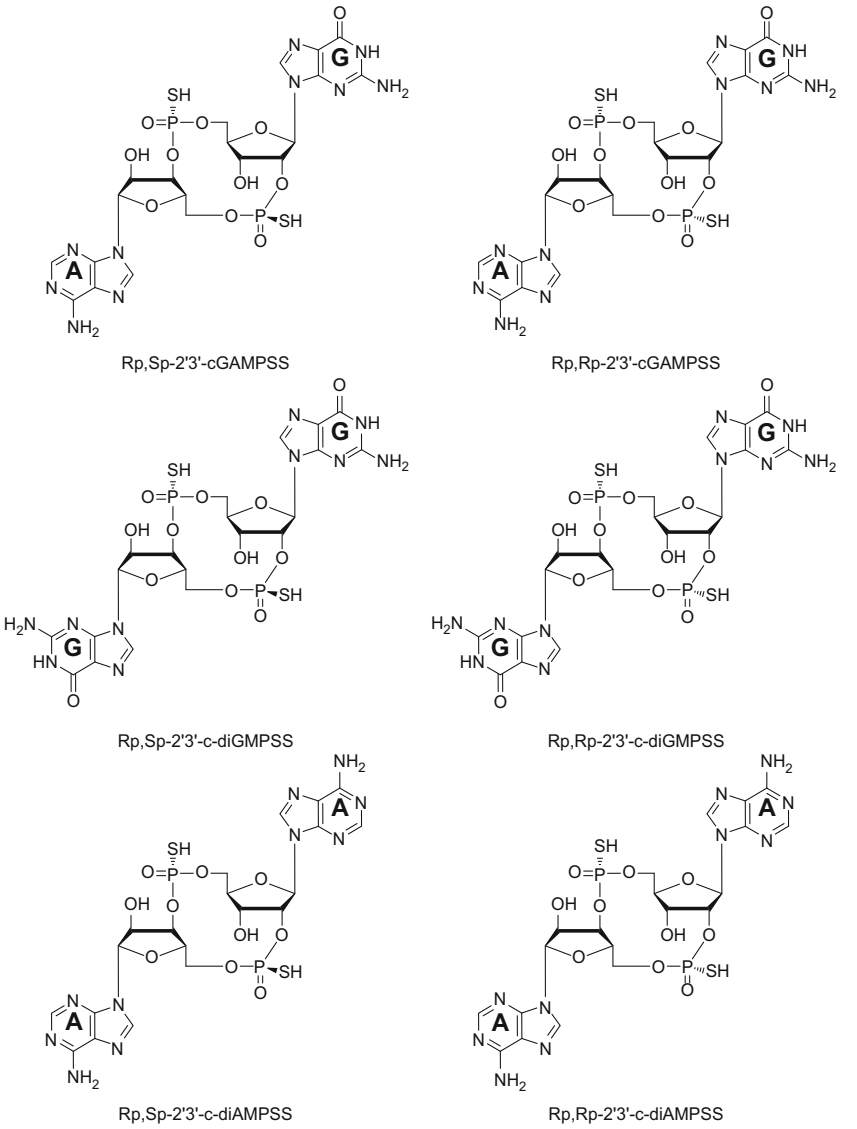


Fig. 6 Chemical structures of stereochemically defined pure isomers of diphosphorothioated noncanonical CDNs

diphosphorothioated noncanonical CDNs were selected for further preclinical development and tested in mice bearing different solid tumor models. Upon intratumoral injection Rp,Rp-2′3′-c-diAMPSS not only significantly reduced tumor growth with a higher therapeutic index compared to Rp,Rp-2′3′-c-diGMPSS but also induced a profound systemic immune response eligible to combat

metastases distant to the injection site. Importantly, the formulation of Rp,Rp-2′3′-c-diAMPSS as adjuvant together with a granulocyte-macrophage colony-stimulating factor (GM-CSF) secreting tumor cell vaccine (STINGVAX(RR-S2-CDA)) produced a strongly enhanced antitumor response in several solid tumor models in mice. This pharmacological effect was significantly higher when compared with the vaccine alone, formulations with canonical 3′3′-c-diAMP (STINGVAX(CDA)), or formulations with other control adjuvants (Fu et al. 2015). In conclusion, Rp,Rp-2′3′-c-diAMPSS was selected as the most promising and first noncanonical CDN for further clinical development as potential anticancer medicine (Corrales et al. 2015).

5 Conclusion and Future Perspectives

The noncanonical CDN 2′3′-cGAMP was identified as an endogenous small second messenger molecule in metazoa in late 2012. It soon became clear that 2′3′-cGAMP has a central function in innate immunity by activating STING leading to subsequent induction of IFN-β, a major pathway to combat cancer or other severe diseases caused by infections with pathogenic bacteria, protozoa, and viruses. Fortunately, a fast adaption of synthetic protocols originally established for canonical CDNs (Gaffney et al. 2010) to 2′3′-cGAMP was possible with commercially available nucleoside phosphoramidite precursors. Thereby sufficient amounts of 2′3′-cGAMP were made available for intensive biological testing of the cGAS-2′3′-cGAMP-STING pathway in several laboratories.

2′3′-cGAMP was modified in different positions (C-8 of guanine, 2′-OH and 3′-OH of riboses) with functional spacers eligible for coupling to agarose beads or for the introduction of reporter groups, like biotin, digoxigenin, and fluorescent dyes. This new toolkit is thought to facilitate the identification of so far unknown STING- or ENPP1-interacting proteins and the quest for new primary receptors of 2′3′-cGAMP signaling. New 2′3′-cGAMP analogs with reporter groups will enable an in-depth biochemical characterization of protein-CDN interactions, as was already proven successful with corresponding bacterial CDNs. 8-Fluo-AET-2′3′-cGAMP, 2′-Fluo-AHC-2′3′-cGAMP, and 3′-Fluo-AHC-2′3′-cGAMP are molecular probes that may be used to establish fluorescence-based binding assays for 2′3′-cGAMP and analogs at receptor proteins. Moreover, analogs like 8-AET-2′3′-cGAMP, 2′-AHC-2′3′-cGAMP, and 3′-AHC-2′3′-cGAMP are convenient starting points for the development of antibodies against 2′3′-cGAMP and for immunoassays to simplify the quantification of 2′3′-cGAMP in biological systems like cells and tissues.

Diphosphorothioate-modified noncanonical CDNs were shown to have significantly improved stability against hydrolysis by ENPP1 in vivo combined with preserved binding affinities and activatory potential for several genotypes of human STING protein. From this class of sulfur-containing CDNs, Rp,Rp-2′3′-c-diAMPSS exhibited pharmacological characteristics justifying further preclinical development work and putative transition into the clinical phase within only 3 years

after the identification of 2′3′-cGAMP. In this context, it will be of high importance to create sophisticated analogs of Rp,Rp-2′3′-c-diAMPSS or related CDNs with modifications in multiple positions of their unique chemical architecture to fully evaluate the pharmacological potency of these new therapeutic lead structures.

Despite enormous achievements that have been accomplished thanks to intensive research during the last years, it should be emphasized that larger collections or libraries of differently modified analogs of 2′3′-cGAMP are only hardly accessible with currently established chemical methods. All "nucleoside approaches" require a minimum of eight chemical steps, rely on available preformed nucleoside phosphoroamidites with several protection groups, and yield only a single or two CDN analogs. It should be noted that most nucleobase modifications or ribose modifications have to be introduced on the nucleoside level often via multiple chemical steps prior to formation of the nucleoside phosphoroamidite. In addition, such modifications have to withstand and should not interfere with reaction conditions of subsequent CDN formation. All these framework conditions are bottlenecks and prohibit the synthesis of libraries of CDN analogs in the midterm. A modification of the antipodal "backbone approach" (Amiot et al. 2006) with alternative common cyclic ribose-2′,5′-3′,5′-diphosphate or cyclic ribose-2′,5′-3′,5′-diphosphorothioate building blocks could be a practicable starting point for the development of parallel syntheses or combinatorial chemistry procedures to generate a larger number of nucleobase-modified noncanonical CDNs. As long as this general drawback has not been overcome by innovative chemical approaches, the design and preparation of new 2′3′-cGAMP analogs will not pick up pace but will require multiple step protocols to obtain single compounds.

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