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# Nucleotidyl cyclase activity of soluble guanylyl cyclase in intact cells

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ABSTRACT

Soluble guanylyl cyclase (sGC) is activated by nitric oxide (NO) and generates the second messenger cyclic GMP (cGMP). Recently, purified sGC $\alpha_1\beta_1$ has been shown to additionally generate the cyclic pyrimidine nucleotides cCMP and cUMP. However, since cyclic pyrimidine nucleotide formation occurred only the presence of $Mn^{2+}$ but not $Mg^{2+}$, the physiological relevance of these *in vitro* findings remained unclear. Therefore, we studied cyclic nucleotide formation in intact cells. We observed NO-dependent cCMP- and cUMP formation in intact HEK293 cells overexpressing sGC $\alpha_1\beta_1$ and in RFL-6 rat fibroblasts endogenously expressing sGC, using HPLC–tandem mass spectrometry. The identity of cCMP and cUMP was unambiguously confirmed by HPLC–time-of-flight mass spectrometry. Our data indicate that cCMP and cUMP play second messenger roles and that $Mn^{2+}$ is a physiological sGC cofactor.



## 1. Introduction

The cyclic purine nucleotides cAMP and cGMP are established second messengers regulating numerous physiological processes, such as relaxation of smooth muscle cells, differentiation and neurotransmission [1,2]. The existence of the cyclic pyrimidine nucleotides cCMP and cUMP in tissues had been postulated [3,4]. Moreover, a specific cytidylyl cyclase and a cCMP-degrading PDE was claimed [5,6]. However, previous methods used to demonstrate the occurrence and generation of cCMP lacked selectivity and sensitivity, resulting in controversial discussion [7]. As a result, very little research has been conducted in the cCMP and cUMP field over the past three decades.

Recently, cCMP and cUMP have been shown to activate PKA with lower potency than, but similar efficacy as, cAMP [8]. cCMP and cUMP are less potent and effective activators of PKG than cGMP [8]. In contrast to cAMP, cGMP and cUMP, cCMP is not cleaved by several human recombinant PDEs [9], pointing to different roles of the various cNMPs in signal transduction. The membrane-permeable cCMP analog dibutyryl-cCMP induces vascular smooth muscle relaxation *via* PKG [10]. Moreover, cCMP and cUMP partially activate the ion channels HCN2 and 4 in recombinant cells and native cardiomyocytes [11]. Furthermore, by using radiometric-, HPLC- and MS approaches, purified bacterial adenylyl cyclase toxins CyaA from *Bordetella pertussis* and edema factor from *Bacillus anthracis* were shown to produce cCMP and cUMP [12]. Lastly, using a highly sensitive and specific HPLC–MS/MS method, purified sGC $\alpha_1\beta_1$ has been shown to produce cCMP and cUMP NO-dependently [13]. Based on these data we developed the hypothesis that cCMP and cUMP play distinct roles as second messenger [14]. However, since cCMP- and cUMP formation by purified sGC occurred only in the presence of $Mn^{2+}$, the physiological relevance of cyclic pyrimidine nucleotide formation by sGC remained unclear. Here, we show that sGC catalyzes cCMP- and cUMP formation also in intact cells.

## 2. Materials and methods

### 2.1. Materials

SNP, IBMX, DEA/NO, ODQ and M7403 medium were purchased from Sigma–Aldrich (Seelze, Germany). Ham's F12, DMEM high glucose 4.5 g/L, penicillin, streptomycin, L-glutamine, and

*Abbreviations:* cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cCMP, cytidine 3′,5′-cyclic monophosphate; cNMP, 3′,5′-cyclic nucleoside monophosphate; cUMP, uridine 3′,5′-cyclic monophosphate; cTMP, thymidine 3′,5′-cyclic monophosphate; cIMP, inosine 3′,5′-cyclic monophosphate; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PDE, phosphodiesterase; pGC-A, particulate guanylyl cyclase A; HCN channel, hyperpolarization-activated cyclic nucleotide-gated ion channel; sGC, soluble guanylyl cyclase; cXMP, xanthosine 3′,5′-cyclic monophosphate; ITP, inosine 5′-triphosphate; NO, nitric oxide; IBMX, 3-isobutyl-1-methylxanthine; ODQ, [1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; DEA/NO, diethylamine NONOate; HPLC–MS/MS, high performance liquid chromatography tandem mass spectrometry; HPLC–MS/TOF, high performance liquid chromatography quadrupole time of flight mass spectrometry; SNP, sodium nitroprusside.

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Dulbecco's PBS was purchased from PAA (Pasching, Austria). Fetal bovine serum was obtained from Lonza (Verviers, Belgium). FuGene was from Roche (Mannheim, Germany) and Zeocin was from Invitrogen (Darmstadt, Germany). HPLC-grade acetonitrile, methanol, and water were supplied by Baker (Deventer, The Netherlands). Tenofovir was used as internal standard for HPLC–MS/MS experiments and was a kind gift from the National Institute of Health, AIDS Research and Reference Program, Division of AIDS (Bethesda, MD, USA). Plasmid vectors pcDNAI/Amp-$\alpha_1$ and pRC/CMV-$\beta_1$ were prepared as described [15].

### 2.2. Cell culture

Cells were grown in a humidified atmosphere of 95% (v/v) air and 5% (v/v) $CO_2$ at 37 °C in DMEM high glucose (4.5 g/L) for HEK293 and Ham's F12 for RFL-6 cells each supplemented with 10% (v/v) fetal bovine serum, 200 μg/mL L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin up to 80% confluency. RFL-6 cells ($5{\cdot}10^5$ cells per 6-well plate) were cultured overnight before stimulation. HEK293 cells ($5{\cdot}10^5$ cells per 6-well plate) were transfected before stimulation with FuGene reagent with 1.5 μg of each plasmid encoding for $\alpha_1$ and $\beta_1$ subunit according the supplier's protocol. 48 h after transfection HEK293 cells were incubated for 10 min with or without IBMX (100 μM) as indicated. SNP was freshly dissolved in 100 mM sodium acetate, pH 5.0, using a light-protected brown tube and added to transfected cells at a final concentration of 100 μM for various times. Reactions were terminated by aspirating the cell culture medium followed by addition of 300 μL cold extraction solution (4 °C) consisting of acetonitrile/methanol/water (2:2:1 (v/v/v)) and 25 ng/mL tenofovir. Cells were scraped off and the suspension was heated for 20 min at 98 °C. After cooling, cell suspension was centrifuged at 20,000*g* for 10 min. The supernatant fluid was evaporated completely under nitrogen atmosphere at 40 °C. Residue was dissolved in 150 μL water and analyzed as described in analysis of cNMPs in intact cells. For determination of protein concentration, cell pellets were dried at room temperature. Dried cell pellets were resolved in 0.1 M sodium hydroxide at 95 °C for 10 min. 10 μL of protein solution were taken for quantitation of protein concentration by means of bicinchoninic acid protein assay. Similar treatment protocols as for HEK293 cells were applied to RFL-6 cells.

### 2.3. Analysis of cNMPs in intact cells

cNMP quantitation was performed via HPLC–MS/MS as described [13] except that separation was performed on an Aglient 1100 series (Waldbronn, Germany) and for detection the QTrap 5500 triple quadrupole mass spectrometer (ABSCIEX, Foster City, CA, USA) was used. Parameters of HPLC–MS/MS fragments are documented in Table S1. Ion source settings and collision gas pressure were manually optimized regarding ion source voltage, ion source temperature, nebulizer gas, and curtain gas (ion source voltage of 5500 V, ion source temperature of 600 °C, curtain gas of 30 psi, collisionally activated dissociation gas of 9 psi). Nitrogen was used as collision gas. Chromatographic data were collected and analyzed with Analyst 1.5.1 software (ABSCIEX). cNMP identification was performed via HPLC–MS/TOF as described for HPLC–MS/MS quantitation, except that separation was performed on a Nexera UHPLC (Shimadzu, Berlin, Germany) and for detection a 5600 TripleTOF (quadrupole/time of flight) mass spectrometer (ABSCIEX) was used. A linear gradient from 100% (v/v) 97/3 (v/v) water/methanol with 50 mM ammonium acetate and 0.1% (v/v) acetic acid to 50% (v/v) 3/97 (v/v) water/methanol with 50 mM ammonium acetate and 0.1% (v/v) acetic acid was applied between 0–5 min followed by re-equilibration of the column at 100% (v/v) 97/3 (v/v) water/methanol with 50 mM ammonium acetate and 0.1% (v/v) acetic acid from 5 to 8 min. Ion source settings were set as followed: ion source voltage floating of 4500 V, ion source temperature of 600 °C, curtain gas of 30 psi. TOF-masses were collected from 50 to 450 Da. Fragment spectra were generated using $3{\cdot}10^6$ cells using a declustering potential of 70 V and a collision energy of 50 ± 20 V. Chromatographic data were collected and analyzed using Analyst 1.5.1 TF and PeakView software (ABSCIEX).

### 2.4. Western blots

HEK293 cells were harvested with 1× PBS 48 h after transfection. Cells were lysed as described in [16]. An amount of 40 μg total protein of cytosolic fraction was separated on a 10% (m/v) SDS–PAGE and transferred on a polyvinylidene fluoride membrane. The individual sGC subunits were detected by using polyclonal antibodies directed against specific epitopes of the $\alpha_1$ subunit (Sigma, Steinheim, Germany) and the $\beta_1$ subunit (Cayman Chemical Company, MI, USA). Detection was performed by the ECL method (Thermo Scientific, IL, USA).

### 2.5. Statistics

Data are presented as means ± SD, and are based on three to six independent experiments. GraphPad Prism software version 5.01 software (San Diego, CA, USA) was used for calculation of mean and SD, as indicated. *p*-Values were calculated by means of ANOVA Bonferroni's multiple comparison test with $^{***}p < 0.001$; $^{**}p < 0.01$; and $^{*}p < 0.05$.

## 3. Results

### 3.1. Identification of cCMP and cUMP in intact cells

In addition to the well-known second messengers cAMP and cGMP we detected the cyclic pyrimidine nucleotides cCMP and cUMP at substantial concentrations in untransfected HEK293 cells and RFL-6 cells using HPLC–MS/MS technology (Table S2). The cNMP concentrations and the cNMP ratios in the two cell types were different, pointing to specific functions of each cNMP (Tables S2 and S3). In HEK293 cells cultured for prolonged time periods (48 h), basal cNMP concentrations decreased, with cCMP and cUMP being more affected than cAMP and cGMP. Using HLPC-MS/TOF, the accurate protonated monoisotopic masses ($[M+H]^+$) of cCMP and cUMP were identified in cells with 306.05 Da and 307.03 Da, respectively (Figs. 1E–H and 2E–H, and Table S4). In addition, fragment spectra of extracts obtained from HEK293 and RFL-6 cells showed the most prominent fragments for cUMP and cCMP (Figs. 1F, H, and 2F, H and Table S4), unequivocally documenting that the detected cNMPs are, indeed, cCMP and cUMP.

### 3.2. NO-stimulated cCMP and cUMP formation in intact transfected HEK293 cells

HEK293 cells transfected with sGC-$\alpha_1\beta_1$ plasmids cells strongly expressed sGC at the protein level (Fig. S1) and exhibited increased basal cGMP concentrations compared to pcDNA3-transfected cells (Table S2). The transfection procedure per se substantially decreased basal cNMP concentrations. HEK293 cells stimulated by the NO-donor SNP [15] and treated with the non-selective PDE inhibitor IBMX [17] exhibited a rapid and pronounced accumulation of cGMP (Fig. 1A). We also observed a transient NO-stimulated cAMP increase, a more delayed cCMP increase and a sluggish but sustained cUMP increase (Figs. 1B–D). The absolute NO-stimulated increases in cAMP, cCMP and cUMP were much smaller than the corresponding cGMP increase. When IBMX was omitted cGMP

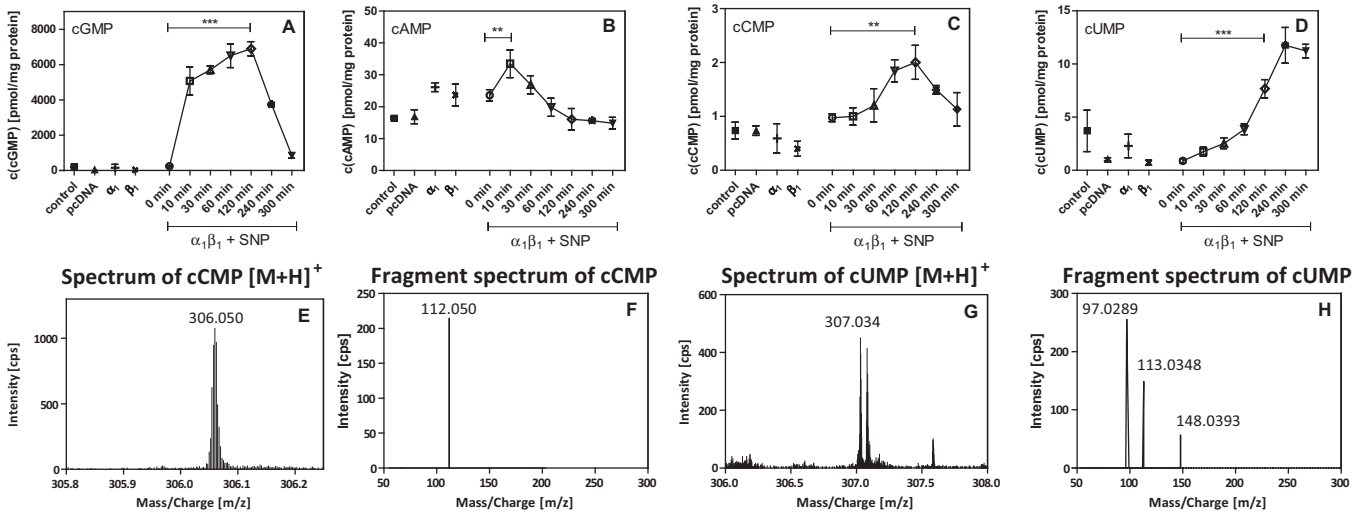


**Fig. 1.** Time-dependent generation of cGMP, cAMP, cCMP, and cUMP by NO-stimulated HEK293 cells transiently overexpressing sGC and verification of cCMP and cUMP by HPLC–MS/TOF. (A–D) Time-dependent generation of cGMP, cAMP, cCMP, and cUMP by NO-stimulated HEK293 cells. 48 h after transfection HEK293 cells were pre-incubated with IBMX (100 μM) for 10 min followed by stimulation with SNP (100 μM). After 0–300 min, cell metabolism was stopped and cNMP concentrations were analyzed by HPLC–MS/MS ($n = 6 \pm$ SD). Please note the different scales of the *y*-axes in all panels. ***$p$-value $\leqslant 0.001$; **$p$-value $\leqslant 0.01$. (E–H) Verification of cCMP and cUMP by HPLC–MS/TOF. 48 h after transfection, cells were preincubated with 100 μM IBMX for 10 min and stimulated with 100 μM SNP for 120 min before analysis. Data shown are representing values of three independent experiments.

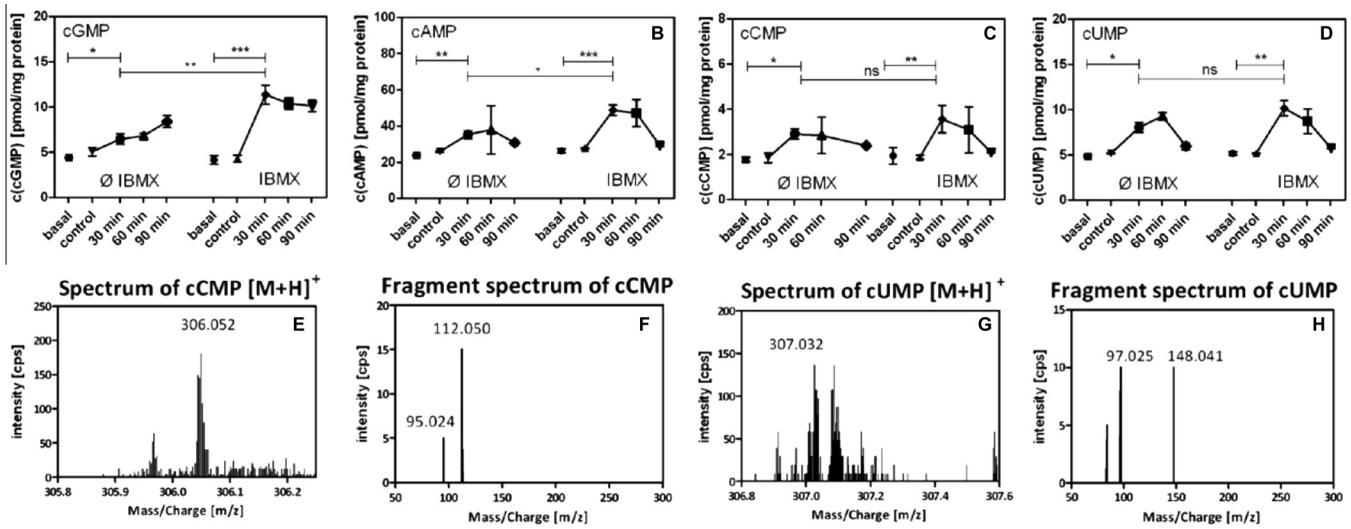


**Fig. 2.** Time-dependent generation of cGMP, cAMP, cCMP, and cUMP by NO-stimulated RFL-6 cells and verification of cCMP and cUMP by HPLC–MS/TOF. (A–D) Time-dependent generation of cGMP, cAMP, cCMP, and cUMP by NO-stimulated RFL-6 cells endogenously expressing sGC ($n = 6 \pm$ SD). At time 0 min (control), cells were stimulated with SNP (100 μM). Ten min prior to stimulation (basal), cells were treated with IBMX (100 μM) or solvent (ø IBMX). Please note the different scales of the *y*-axes in all panels. ***$p$-value $\leqslant 0.001$; **$p$-value $\leqslant 0.01$. (E–H) Verification of cCMP and cUMP by HPLC–MS/TOF. Cells were seeded in 6-well plates for 24 h with $5 \cdot 10^5$ cells per well before analysis. Data shown are representative of three independent experiments.

and cAMP levels significantly decreased but cCMP and cUMP concentrations were not influenced (Table S5), indicative of different PDE inactivation mechanisms of the various cNMPs [9]. The sGC inhibitor ODQ [15] abolished NO-stimulated cNMP accumulation in HEK293-$\alpha_1\beta_1$ cells (Table 1). Most notably, ODQ almost abrogated cCMP levels in HEK293-$\alpha_1\beta_1$ cells, suggesting that sGC also plays a role in maintaining basal cellular cCMP concentrations.

### 3.3. NO-stimulated cCMP and cUMP formation in intact RFL-6 cells

RFL-6 cells express sGC endogenously [18]. Stimulation of RLF-6 cells with SNP in the absence or presence of IBMX resulted in a time-dependent accumulation of intracellular cGMP (Fig. 2A). Additionally, NO-stimulated increases in cAMP, cCMP and cUMP were observed (Fig. 2B–D). The cyanide-free NO-donor DEA/NO also increased all four cNMPs in RFL-6 cells (Table 2). Compared

**Table 1**
Inhibition of cNMP accumulation in HEK293 sGC $\alpha_1\beta_1$ cells by ODQ. HEK293 cells were transiently transfected with plasmids encoding for rat sGC $\alpha_1\beta_1$. 48 h after transfection cells were preincubated for 10 min with 100 μM IBMX and then stimulated with 100 μM SNP with or without 100 μM ODQ. After 120 min cell metabolism was stopped and cNMP concentrations were analyzed. Concentrations are given as mean values ± SD based on six independent experiments in pmol/mg protein.

| Compound | cGMP | | cAMP | cUMP | | cCMP | |
|---|---|---|---|---|---|---|---|
| None | 17.8 ± 8.9 | ]*** | 15.4 ± 1.0 | 0.8 ± 0.4 | ]*** | 0.4 ± 0.3 | ]*** |
| SNP | 4,888 ± 899 | ]*** | 16.3 ± 0.9 | 7.9 ± 2.8 | ]*** | 1.4 ± 0.8 | ]*** |
| SNP + ODQ | 12.5 ± 8.1 | ] | 15.9 ± 1.3 | 0.6 ± 0.1 | ] | 0.02 ± 0.01 | ] |

***$p$-value $\leqslant 0.001$.

**Table 2**
Stimulation of RLF-6 cells by SNP and DEA/NO. RFL-6 cells were seeded for 24 h with $5{\cdot}10^5$ cell per well in one 6-well plate. Before stimulation with 100 μM SNP or DEA/NO for 30 min cells were preincubated by IBMX (100 μM) for 10 min. Concentrations are given as mean values ± SD based on six independent experiments.

| Stimulator | cGMP | cAMP | cUMP | cCMP |
|---|---|---|---|---|
| None | 3.24 ± 1.68 | 21.08 ± 1.23 | 4.88 ± 0.83 | 1.85 ± 0.28 |
| SNP | 14.04 ± 0.91*** | 33.18 ± 0.70*** | 9.50 ± 1.04** | 3.64 ± 0.40** |
| DEA/NO | 12.64 ± 2.01** | 31.87 ± 1.62*** | 8.86 ± 0.60** | 3.10 ± 0.37* |

*** $p$-value ⩽ 0.001.
** $p$-value ⩽ 0.01.
* $p$-value ⩽ 0. 05.

to HEK293-$\alpha_1\beta_1$ cells, the contribution of cyclic pyrimidine nucleotides to total cNMP formation in RFL-6 cells was relatively high.

## 4. Discussion

Early studies in the mid 1980s tentatively identified cCMP in tissues using fast atom bombardment MS [3,4], but specificity and sensitivity of the method are insufficient for unequivocal identification as compared to modern MS analytical methods. Specificity of cCMP antibodies is also problematic [19]. In the present study, we took advantage of the most advanced HPLC–MS/MS and HPLC–MS/TOF methods currently available to detect cNMPs. In this study, the cyclic pyrimidine nucleotides cCMP and cUMP were unambiguously detected in substantial concentrations in representative mammalian cell lines besides cAMP and cGMP.

The identification of cCMP and cUMP in intact cells raises the question which enzyme is responsible for this cNMP formation. There is no evidence for a role of pGC-A in this process [20], but highly purified GC generates cCMP and cUMP in a NO-dependent manner [13]. However, purified sGC generates cCMP and cUMP only in the presence of $Mn^{2+}$, but not in the presence of $Mg^{2+}$ [13]. Because cellular $Mg^{2+}$ concentrations are much higher than $Mn^{2+}$ concentrations [21], we were skeptical about a role of sGC in the regulation of cCMP and cUMP levels in intact cells. Nonetheless, we clearly detected NO-stimulated and ODQ-inhibited cCMP and cUMP formation in cells endogenously expressing sGC and cells transiently overexpressing sGC. Hence, sGC regulates not only cellular cGMP- but also cCMP- and cUMP levels. These data also confirm our hypothesis that in intact cells, $Mn^{2+}$ rather than $Mg^{2+}$ is the relevant cofactor for sGC [14]. Thus, our study will also stimulate research on the physiological role of $Mn^{2+}$, a relatively poorly addressed field so far [14,21]. Based on the present sGC data with intact cells, it is also likely that the bacterial toxins edema factor and CyaA generate cCMP and cUMP in intact cells [12].

Compared to cells overexpressing sGC, cells endogenously expressing sGC revealed relatively large increases in cCMP and cUMP. The absolute basal and NO-stimulated cGMP- and cUMP levels in RFL-6 cells are very similar. These findings argues against the notion that cUMP is a trivial by-product of a leaky sGC. The cell type-specific differences in NO-dependent cCMP and cUMP formation may be due to different hydrolysis and/or export rates of cNMPs. Basal cCMP formation in cells may also be mediated by sGC considering the fact that the sGC inhibitor ODQ effectively inhibits basal cCMP formation by purified sGC [13] and almost abolishes cCMP levels in intact cells.

The MS methods used in the study are very well suited for unequivocal cNMP identification (HPLC–MS/TOF) and cNMP quantitation (HPLC–MS/MS) in a cell population. However, MS is not feasible for analysis of spatiotemporal cNMP patterns in cells so far, although advances in metabolomic MS imaging have been described [22]. Currently, for spatiotemporal analyses, fluorescence sensor-based methods are the most feasible approach. For cGMP and cAMP, fluorescence-based detection methods are available [23], and studies with such sensors have revealed substantial spatiotemporal differences in cNMP levels [24]. Thus, relatively small cNMP- and cUMP changes observed over a large cell population as assessed by traditional MS methods do not exclude the possibility that in individual cells or cell compartments at a specific time point, large cCMP- and cUMP increases do occur. Accordingly, the next major challenge in the cCMP- and cUMP field is the development of highly sensitive and specific fluorescence sensors. As a first step, specific cCMP- and cUMP-binding proteins have to be identified. Appropriate biochemical analysis methods are already in place [25]. As an alternative approach, specific cCMP- and cUMP-binding proteins may be engineered. This is not an impossible task considering that even naturally occurring uracil base-specific receptors exist [26]. In this context, it will also be important to identify cCMP and cUMP not only in cultured mammalian cell lines but also in primary mammalian cells and intact organs. We predict that such analyses will demonstrate the presence of cCMP and cUMP in native vertebrate systems. In the nematode *Caenorhabditis elegans*, cCMP and cUMP were not identified [27], suggesting that these cyclic nucleotides are evolutionary relatively new molecules.

The different NO-stimulated and ODQ-inhibited cNMP patterns in terms of absolute and relative magnitude and time course reported herein suggest distinct functional roles of cGMP, cCMP and cUMP. Therefore, it will be necessary to dissect cellular effects of these cNMPs in numerous cell systems. For this formidable task, the application of membrane-permeant acetoxymethylesters of cNMPs [28] and label-free sensor techniques [29] are feasible approaches. For chemical reasons, the more commonly used butyrate ester approach cannot be applied to cUMP [10,30]. Noteworthy in this context is the fact that cGMP-independent effects of sGC have been reported before [31–33], but it is unknown whether cCMP and/or cUMP are involved in these effects.

In conclusion, this study unambiguously demonstrated the presence of cCMP and cUMP in intact cultured mammalian cells using HPLC–MS/TOF. sGC generates cCMP and cUMP not only *in vitro* but also in intact cells. An implication of our data is that $Mn^{2+}$ is a physiological cofactor for sGC. Our data provide first hints for distinct cellular functions of cGMP, cCMP and cUMP and we have discussed some future directions of research in this exciting new field of signal transduction.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.108.

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