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cNMP-AMs mimic and dissect bacterial nucleotidyl cyclase toxin effects

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ABSTRACT

In addition to the well-known second messengers cAMP and cGMP, mammalian cells contain the cyclic pyrimidine nucleotides cCMP and cUMP. The *Pseudomonas aeruginosa* toxin ExoY massively increases cGMP and cUMP in cells, whereas the *Bordetella pertussis* toxin CyaA increases cAMP and, to a lesser extent, cCMP. To mimic and dissect toxin effects, we synthesized cNMP-acetoxymethylesters as prodrugs. cNMP-AMs rapidly and effectively released the corresponding cNMP in cells. The combination of cGMP-AM plus cUMP-AM mimicked cytotoxicity of ExoY. cUMP-AM and cGMP-AM differentially activated gene expression. Certain cCMP and cUMP effects were independent of the known cNMP effectors protein kinases A and G and guanine nucleotide exchange factor Epac. In conclusion, cNMP-AMs are useful tools to mimic and dissect bacterial nucleotidyl cyclase toxin effects.

1. Introduction

cAMP and cGMP are well-established second messengers [1,2]. In addition to these cyclic purine nucleotides, mammalian cells contain the cyclic pyrimidine nucleotides cCMP and cUMP [3,4]. Soluble adenylyl cyclase plays a key role in maintaining basal cNMP concentrations in mammalian cells [4]. The *Pseudomonas aeruginosa* nucleotidyl cyclase toxin ExoY induces massive increases in cGMP and cUMP in mammalian cells and smaller increases in cAMP and cCMP [5]. In contrast, the *Bordetella pertussis* nucleotidyl cyclase toxin CyaA massively increases cAMP and, to a lesser extent, cCMP [5]. ExoY induces lung damage and necrosis of cells [5,6]. However, given the fact that ExoY increases the levels of all four cNMPs, it is impossible to answer the question what the contribution of any given cNMP to the biological toxin effect is. In order to address this problem, we studied the set of four cNMP-AMs shown in Fig. 1. In cNMP-AMs, the hydrophilic phosphate group is protected by an acetoxymethylester so that the compounds can penetrate the plasma membrane [7]. Within the cells, the cNMP moiety is released and induces biological effects. As control compound, we used PO_4-AM_3. Here, we show that cNMP-AMs are useful experimental tools to mimic and dissect bacterial nucleotidyl cyclase toxin effects.

2. Materials and methods

2.1. Materials

Rp-cAMPS, Rp-8-Br-cAMPS, Rp-8-Br-PET-cGMPS, Rp-8-pCPT-cGMPS, 8-pCPT-2′-O-Me-cAMP, cNMPs and PO_4-AM_3 were obtained from Biolog LSI (Bremen, Germany).

Abbreviations: AM, acetoxymethylester; 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; DMR, dynamic mass redistribution; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; Epac, exchange protein directly activated by cAMP; PO_4-AM_3, phosphate tris(acetoxymethyl)ester; Rp-8-Br-cAMPS, (Rp)-8-bromoadenosine-3′,5′-cyclic monophosphorothioate; Rp-cAMPS, (Rp)-adenosine-3′,5′-cyclic monophosphorothioate; Rp-8-Br-PET-cGMPS, (Rp)-β-phenyl-1, N^2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate; Rp-8-pCPT-cGMPS, (Rp)-8-(*para*-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate; 8-pCPT-2′-O-Me-cAMP, 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate; RT-PCR, real-time PCR; EBAO, ethidium bromide acridine orange.

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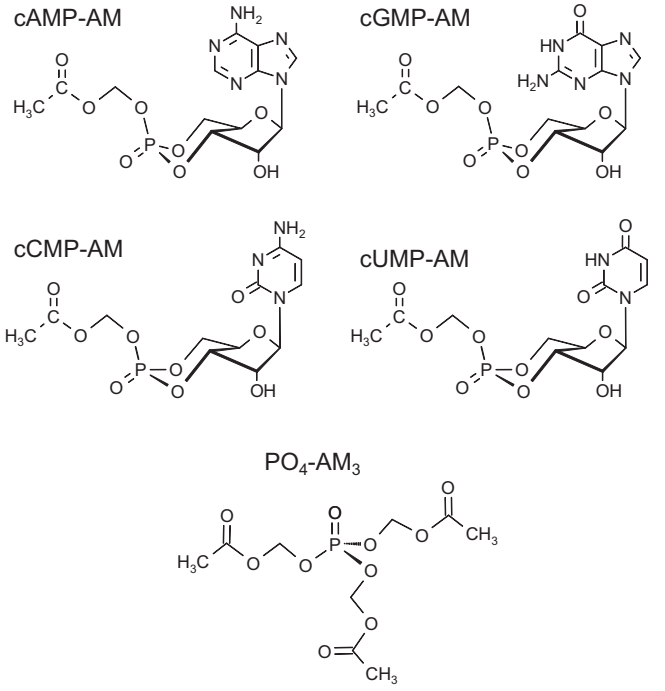

Fig. 1. Structures of cNMP-AMs and PO_4-AM_3. The AM group neutralizes the negative charge of the cNMP phosphate group. Accordingly, AM compounds can penetrate the plasma membrane. In the cytosol, esterases cleave the AM compounds, releasing the free cNMP and phosphate, respectively. Please note that PO_4-AM_3 contains three AM groups. Accordingly, in experiments, PO_4-AM_3 is used at threefold lower concentrations than cNMP-AMs. In order to avoid cleavage of AM compounds by extracellular esterases, experiments should be performed in the absence of serum supplementation.

2.2. Synthesis of cCMP-AM and cUMP-AM

All chromatographic experiments were performed at ambient temperature. The analytical HPLC-system consisted of a L 6200 pump, a L 4250 variable wavelength UV/Vis-detector, and a D 7500 chromato-integrator (all Merck-Hitachi, Darmstadt, Germany). The stationary phases were YMC ODS-A 12 nm, S-11 μm (YMC, Dinslaken, Germany) or Kromasil 100-10, RP-8 (Eka Nobel, Bohus, Sweden) in 250 × 4.6 mm stainless steel columns with Gemini C18, 4 × 3 mm Security guard columns (Phenomenex, Aschaffenburg, Germany). Semipreparative HPLC was performed with a LC-8A preparative liquid chromatograph (Shimadzu, Duisburg, Germany), a preparative K 2001 UV-detector (Knauer, Berlin, Germany), a L200E analog recorder (Linseis, Selb, Germany), and either YMC ODS-A 12 nm, S-11 μm (YMC) as stationary phase in a 250 × 20 mm stainless steel column (CS-Chromatography Service, Düren, Germany). Mass spectra were recorded with an Esquire LC 6000 spectrometer (Bruker Daltonics, Bremen, Germany) in the ESI-MS mode with 50/49.9/0.1 (v/v/v) propanol-2/water/formic acid as matrix. UV-spectra for preparation of aliquots were recorded with a Helios β-spectrometer (Spectronic Unicam, Leeds, UK) in aqueous phosphate buffer, pH 7. All reagents were of analytical grade or the best grade available from commercial suppliers.

2.2.1. cCMP-AM

220 μmol cCMP (diisopropylethylammonium salt) were carefully dried and suspended in 10 mL acetonitrile. After addition of 1100 μmol (110 μL; 5 equivalents) acetoxymethyl bromide and 1320 μmol (305 mg; 6 equivalents) Ag_2O, the reaction mixture was stirred vigorously at ambient temperature for 35 min. Progress of AM-ester formation was monitored by analytical HPLC with 16% (v/v) acetonitrile, 20 mM triethylammonium formate (pH 6.80) as eluent. After reaction was completed, solid Ag_2O was removed by filtration through a 0.2 μm PTFE membrane, and 2 mL aliquots of the raw mixture were evaporated under reduced pressure with oil pump vacuum. The residue was redissolved in DMF (~2–3 mL) and purified by semipreparative HPLC using 15% (v/v) acetonitrile as eluent. Product fractions were evaporated under reduced pressure to produce 84.5 μmol cCMP-AM as mixture of axial and equatorial isomers with a purity of >99.5% (yield: 38.4%). Formula: $C_{12}H_{16}N_3O_9P$ (MW: 377.2); ESI-MS pos. mode: *m/z* 378 $(M + H)^+$, *m/z* 479 $(M + H + TEA)^+$; neg. mode: *m/z* 376 $(M - H)^-$, *m/z* 304 $(M - AM - H)-$; UV–VIS (pH 7.0) λ_{max} 270 nm (ε = 9000).

2.2.2. cUMP-AM

Synthesis and work-up of cUMP-AM was performed in parallel reactions with 3 × 500 μmol cUMP (silver salt) and 2500 μmol (250 μL; 5 eq.) acetoxymethyl bromide in 30 mL acetonitrile as described for cCMP-AM. The reaction was monitored by analytical HPLC (Kromasil) with 15% (v/v) acetonitrile, pH 5 (0.25 μL acetic acid per 1 L), and the raw product was purified by semipreparative HPLC (Kromasil) with 5–10% (v/v) acetonitrile. Product-containing fractions were evaporated *in vacuo* and 110.7 μmol cUMP-AM were obtained as a mixture of isomers with a purity of 98.78% (yield: 7.4%). Formula: $C_{12}H_{15}N_2O_{10}P$ (MW: 378.2); ESI-MS pos. mode: *m/z* 401 $(M + H + Na)^+$, *m/z* 379 $(M + H)^+$; neg. mode: *m/z* 305 $(M - AM - H)^-$, *m/z* 377 $(M - H)^-$; UV–VIS (pH 7.0) λ_{max} 260 nm (ε = 10000).

2.3. DMR measurements

For DMR measurements a beta version of the Corning Epic biosensor (Corning, Corning, NY, USA) or the Enspire multimode reader (Perkin Elmer, Hamburg, Germany) that contains an integrated DMR module was used. Each well of the DMR biosensor microplate contains a grating biosensor that guides polarized broadband light through the bottom of the plate generating an electromagnetic field that extends 150 nm into the cell layer. As a result of cellular response, relocation of intracellular constituents leads to a local change of refraction index that is translated into a wavelength shift (in pm) of the reflected light. The magnitude of this wavelength shift is proportional to the amount of DMR. Increase of mass contributes positively and decreases negatively to the overall response. The resulting optical signatures reflect cellular processes such as shape change, cytoskeletal reorganization or cellular adhesion as a consequence of engagement of intracellular signaling cascades.

DMR measurements were performed as described [8]. In brief, HEK293 and B103 cells were seeded into 384-well fibronectin coated DMR biosensor microplates with a density of 15,000 cells per well and grown overnight (at 37 °C and 5% (v/v) CO_2) to confluent monolayers. Esterases present in the serum supplements of the cell culture medium can degrade the test compounds by hydrolysis of esters and, therefore, strongly reduce cell-loading efficiency. Hence, after removal of medium cells were washed at least twice with HBSS containing 20 mM HEPES (DMR buffer) to ensure absence of serum supplements, and a residual volume of 30 μL of DMR buffer was left in each well. Immediately before DMR registration test compounds were prepared at 4× final concentration in pre-warmed DMR buffer and placed into the compound source plate. Then, the sensor plate was scanned and a baseline optical reading was recorded. Finally, 10 μL of compound solutions were

transferred into the sensor plate and DMR was monitored for at least 9000 s.

2.4. Other methods

cNMP quantitation in cells was performed via HPLC–MS/MS as described using a QTrap5500 triple quadrupole mass spectrometer (ABSCIEX, Foster City, CA, USA) [9,10]. Cell culture and B103 cell transfection with ExoY plasmid was performed as described [3–5]. Cell viability was assessed using light microscopy and EABO staining [11]. FACS analysis of cell viability was performed as described [5]. Guanine nucleotide exchange factor activity of Epac1 on Rap1B was determined with the fluorescent GDP analog 2′,3′-O-(N-methylanthraniloyl)-GDP as described [12]. Gene expression in B103 cells was performed using the "Whole Rat Genome Oligo Microarray 4 × 44 Kv2″ (Agilent, Böblingen, Germany) containing 45,220 oligonucleotide probes. For RT-PCR studies, HEK293 cells were incubated with different compounds for 1 h, followed by TaqMan probe analysis for c-fos using the ΔΔCt method [11].

2.5. Statistics

Data are presented as means ± SD, and are based on 4–8 independent experiments.

3. Results

In B103 neuroblastoma cells, ExoY induces massive increases in cGMP and cUMP and necrosis [5]. cGMP-AM (200 μM for 4 h) increased cGMP levels in B103 cells to a similar extent as did a 4-h treatment of cells with *P. aeruginosa* expressing ExoY (Fig. 2) [5]. By analogy, cUMP-AM (200 μM for 4 h) was similarly effective at increasing cUMP as a 4-h treatment of cells with *P. aeruginosa* expressing ExoY (Fig. 2) [5]. The combination of cGMP-AM and cUMP-AM yielded similar levels of the cognate cNMPs as treatment with the single cNMP-AM. cGMP-AM and cUMP-AM exhibited no effect on cAMP and cCMP levels. cUMP-AM induced a small cGMP increase which may be due to blockade of a cGMP-degrading phoshopdiesterase by cUMP. The control compound PO_4-AM_3 had no effect on cNMP levels. The uptake of cNMP-AMs into cells was very rapid, i.e. already after 5 min significant levels of the free cognate cNMP were reached, and levels remained elevated at least for 6 h (later time points were not studied) (Fig. S1). In contrast, non-cognate cNMP levels remained low.

Under control conditions B103 cells grew as monolayer with neurite extensions (Fig. 3A–C) [11]. Green color in the EBAO staining is indicative for cell viability. cGMP-AM had little effect on cell morphology and viability (Fig. 3D–F). In contrast, cUMP-AM induced marked cell clustering but no marked decrease in cell viability (Fig. 3G–I). The combination of cGMP-AM and cUMP-AM resulted in detachment of the cells from the surface and a massive decrease in viability as evident by change in cell staining from green to red (Fig. 3J–L). Analysis of cell viability by FACS [5] revealed an increase in the percentage of necrosis from ~2–3% in control cells or cells treated with either cGMP-AM or cUMP-AM alone to 54% in cells treated with the combination of cGMP-AM plus cUMP-AM (data not shown). Transfection of B103 cells with ExoY induced similar morphological changes as treatment with cGMP-AM plus cUMP-AM. Specifically, we observed cell rounding and cell clustering and an increase in increase in the percentage of necrotic and apoptotic cells (Fig. S2D–F). These data fit to the recently reported FACS analysis data [5]. In contrast, transfection of B103 cells with the catalytically inactive ExoY mutant K81M did not result in cell rounding, cell clustering, apoptosis and necrosis (Fig. S2A–C).

Morphological and viability changes in B103 cells following exposure to cGMP-AM and cUMP-AM were accompanied by changes in gene expression after a 2 h incubation (Fig. S3). We conducted a microarray analysis encompassing ⩾45,000 genes. Somewhat unexpectedly, we found that expression of only few genes changed significantly. Specifically, cGMP-AM significantly increased expression of the cyclic-dependent kinase inhibitor 1 (cdkn1a, regulator of cell cycle progression), early response gene 1 (egr1, promoting neuronal differentiation and neurite growth), egr2 and inducible heme oxigenase 1 (hmox1, a redox gene usually elevated by heavy metals, endotoxin and oxidizing compounds). cUMP-AM increased expression of cdkn1a, egr1, egr2, growth differentiation factor 15 (gdf15, a neuroprotective and neurotrophic factor) and small GTPase inhibitor of RhoA (rnd1, promoting neuronal differentiation and neurite growth). cUMP-AM was more effective at inducing expression of cdkn1a, egr1 and rnd1 than cGMP-AM, whereas the opposite was true for hmox1. Collectively, these data show that cGMP-AM and cUMP-AM alter expression of few genes associated with cell cycle, cell differentiation and cell stress. The selective effects of cGMP-AM and cUMP-AM on the expression of only few genes argues against non-specific effects of the cNMPs.

We also addressed the question whether cNMP-AMs are feasible for dissecting the mechanisms underlying the biological effects

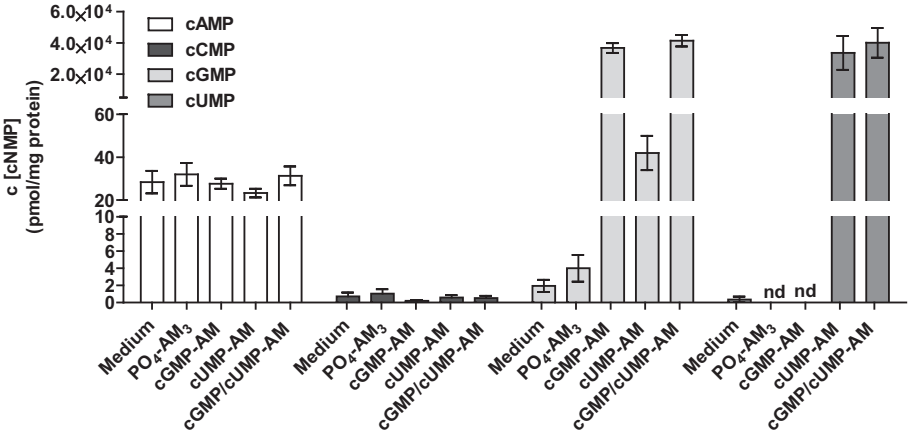

Fig. 2. cGMP- and cUMP-uptake of B103 cells after cGMP-AM and cUMP-AM incubation. 4 × 10^5 B103 cells were incubated for 24 h in low-serum medium and then treated with 200 μM cGMP-AM and cUMP-AM alone or with a 1:1 mixture of both (each 200 μM final) at 37 °C for 4 h. PO_4-AM_3 66 μM and 132 μM served as analog control. Subsequently, cells were processed for HPLC–MS/MS quantitation of cNMPs. nd: not detected.

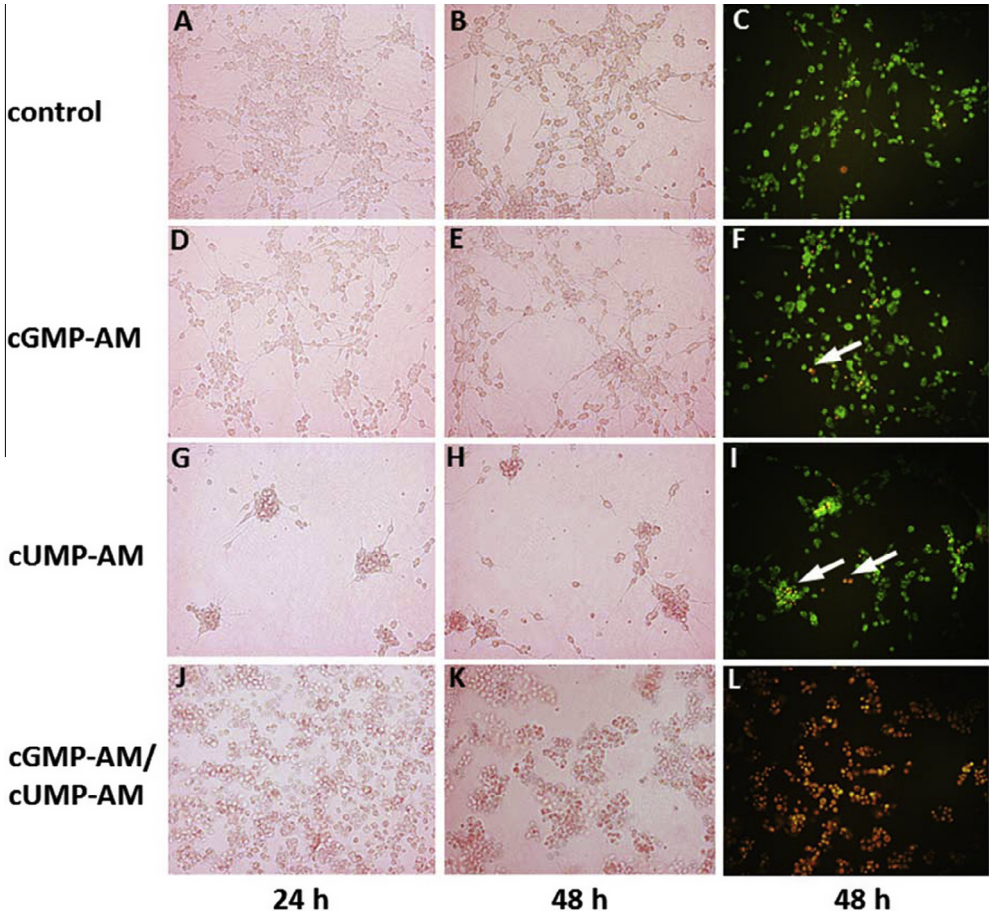

Fig. 3. ExoY mimicry by cGMP-AM and cUMP-AM in B103 cells. B103 cells were incubated with 200 μM of cGMP-AM, cUMP-AM and a 1:1 mixture of cGMP-AM/cUMP-AM, respectively. PO_4-AM_3 in a concentration of 132 μM served as control. C, F, I and L represent EBAO stained cells. All other panels represent unstained cells. White arrows indicate representative apoptotic/necrotic cells. The experiment was conducted at least three times. Green = viable cells, red = apoptotic/necrotic cells. Magnification, 200-fold. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of individual cNMPs. For these studies we used the holistic DMR assay that has already been very successfully applied with regard to the dissection of signaling pathways activated by G protein-coupled receptors [8]. The DMR assay is very sensitive in terms of picking up cell signals, and therefore, we hoped that we could use cNMP-AMs at lower concentrations than for mimicry of ExoY effects (Figs. 2, 3, S2 and S3) [5]. Such a procedure would facilitate studies with inhibitors of known cNMP targets, i.e. PKA and PKG because such inhibitors compete with intracellular cNMPs for binding to kinases [13]. cAMP-AM up to 100 μM had no effect on DMR in HEK cells (Fig. 4A). cGMP-AM exhibited only a small and delayed signal (Fig. 4B). In marked contrast, cCMP-AM induced large and sluggish DMR signals, reaching saturation between 50 and 100 μM (Fig. 4C). cUMP-AM (100 μM) induced substantial larger DMR signals than cCMP-AM (Fig. 4D). In contrast to cCMP-AM, cUMP-AM also exhibited a pronounced rapid DMR response phase. In addition, the cUMP-AM response was not yet saturated at 100 μM. Control experiments revealed that PO_4-AM_3, the non-esterified cNMPs themselves and the membrane-permeable Epac activator 8-pCPT-2′-O-Me-cAMP (up to 300 μM) had no effect on DMR responses (data not shown). A combination of PKG inhibitors suppressed the responses of cUMP-AM more effectively than a combination of PKA inhibitors (Fig. 4E). The addition of PKA inhibitors on top of PKG inhibitors exhibited no additional inhibitory effect. Whereas PKA inhibitors strongly inhibited the DMR response of cCMP-AM, PKG inhibitors potentiated the cCMP response and unmasked a rapid signature (Fig. 4F). Addition of PKA inhibitors on top of PKG inhibitors had no additional effect.

Similar to the observations made for HEK293 cells, cCMP-AM induced a delayed DMR response in B103 cells (Fig. S4). Again, PKA inhibitors strongly reduced the cCMP-AM signal, whereas PKG inhibitors unmasked a rapid cCMP-AM response. PKA inhibitors had no effect in the presence of PKG inhibitors. In HEK293 cells, cCMP-AM slightly increased c-Fos expression (Fig. S5). The effect of cCMP-AM on c-Fos expression was strongly potentiated by a PKA or PKG inhibitor and a combination of both inhibitors. Finally, we assessed the effects of cCMP and cUMP on the activity of purified Epac. cCMP up to 100 μM, in contrast to cAMP, had virtually no stimulatory effect on Epac, and even an increase of cCMP of up to 1 mM resulted only in a minimal response (Fig. S6). cUMP was ineffective at activating Epac.

4. Discussion

cNMP-AMs enter cells and release the cNMP into the cytosol following cleavage of the ester. Sustained and high cNMP levels result, with no marked evidence for cross-regulation of other cNMPs by a given cNMP-AM. The control compound PO_4-AM_3 is also inert with respect to cNMP increases. The combination of cGMP-AM and cUMP-AM mimics the effects of the *P. aeruginosa* nucleotidyl cyclase toxin ExoY with respect to intracellular cGMP and cUMP levels and cytotoxicity [5]. The combination of both

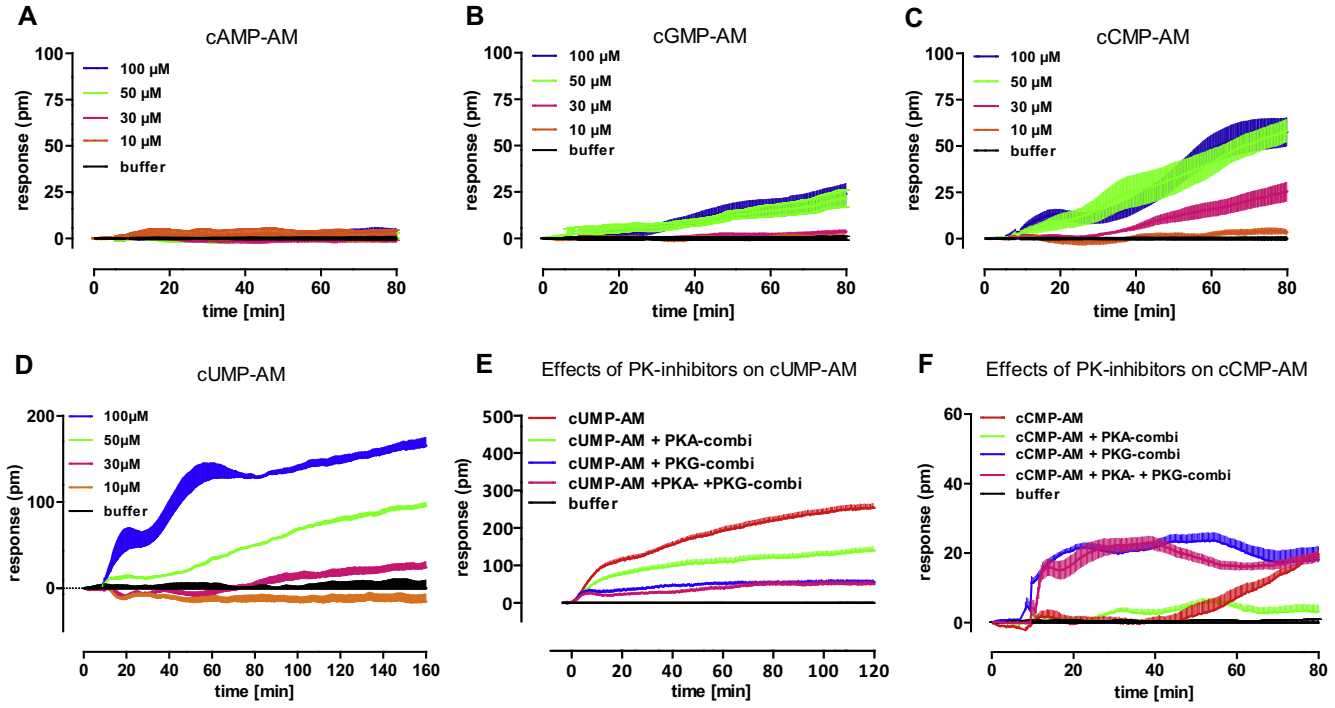

Fig. 4. DMR responses in HEK293 cells treated with cNMP-AMs. DMR tracings of HEK293 cells treated with cAMP-AM (A), cGMP-AM (B), cCMP-AM (C), and cUMP-AM (AM) (D) for 80 min. (E) HEK293 cells treated with 50 µM cUMP-AM in the presence or absence of PKA inhibitors (500 µM Rp-8-Br-cAMPS and 500 µM Rp-cAMPS) and/or PKG inhibitors (300 µM Rp-8-Br-PET-cGMPS and 500 µM Rp-8-pCPT-cGMPS). (F) HEK293 cells treated with 10 µM cCMP-AM in the presence or absence of PKA inhibitors (500 µM Rp-8-Br-cAMPS and 500 µM Rp-cAMPS) and/or PKG inhibitors (300 µM Rp-8-Br-PET-cGMPS and 500 µM Rp-8-pCPT-cGMPS). Traces are corrected for the effects of the inhibitors alone in the absence of cNMP-AMs.

cNMP-AMs was required for toxin mimicry. cGMP and cUMP are functionally not equivalent because only cUMP-AM induced morphological changes alone and moreover, cGMP-AM and cUMP-AM exhibited differential effects on gene expression.

cCMP-AM and cUMP-AM induce pronounced DMR responses. The responses were observed at low compound concentrations (10–100 µM) and were not mimicked by cAMP-AM, cGMP-AM, PO_4-AM_3 or non-esterified cNMPs. All these data indicate the DMR responses by cCMP-AM and cUMP-AM are specific for the intracellularly released cNMP. Dissection between effects of cCMP and cUMP *versus* cAMP and cGMP is critical for postulating a second messenger role of the former cNMPs. Both, the effects of cCMP and cUMP are partially mediated by PKA as revealed by the partial inhibitory effects of PKA inhibitors. The EC_{50} values of cCMP and cUMP for PKA and PKG activation [14] fit well to the concentrations required for induction of DMR responses by cNMP-AMs. cCMP and cUMP are also partial activators of purified PKG [14], and modulation of DMR responses of cCMP-AM and cUMP-AM by PKG inhibitors is consistent with a role of PKG in cell responses. However, while in the case of cUMP, PKG plays a stimulatory role, in case of cCMP, PKG exerts a tonic inhibition that is released by PKG inhibition and dominates the PKA regulation. These data also indicate that cCMP and cUMP act *via* different mechanisms. This interpretation is supported by the different cCMP and cUMP kinetics. An implication of these data is that ExoY from *P. aeruginosa* (predominantly increasing cUMP) and CyaA from *B. pertussis* (predominantly increasing cCMP) [5] manipulate mammalian signal transduction pathways in different manners and address distinct intracellular targets. Furthermore, there is no evidence for a role of Epac in DMR responses. Most importantly, the persistence of cCMP-AM DMR and gene expression responses in the presence of PKA and PKG inhibitors and the inefficiency of cCMP and cUMP at activating Epac indicate that additional effector proteins for these cNMPs exist.

In conclusion, the present study has made an important contribution towards elucidation of the pathophysiological effects of ExoY and different second messenger functions of cCMP and cUMP. cGMP and cUMP, can be clearly dissociated from each other, and cCMP and cUMP induce distinct cellular responses that cannot be explained by the known cNMP effector proteins PKA, PKG and Epac. The next two steps in the elucidation of the second messenger functions of cCMP and cUMP are straightforward. First, we need to identify the specific binding proteins of cCMP and cUMP. The appropriate methodologies are already in place [15]. Second, the research community is invited to follow the paradigm outlined in this study and examine the biological effects of cNMP-AMs alone and in combination in multiple systems to understand the (patho)physiological roles of cUMP and cCMP. A recent study revealed that cCMP and cUMP are present in numerous mammalian cell culture lines and primary mammalian cells and that any given cell type possesses a unique cNMP pattern [16]. Based on these data it can be assumed that cCMP and cUMP play important biological roles in many systems. These cNMP roles can be unmasked with cNMP-AMs. Lastly, it should be kept in mind that it is not only important to add a single cNMP-AM to cells. Rather, cNMP-AM combinations are critical, mimicking intracellular cNMP patterns.

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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.2014.07.134.

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