Biochemical and Biophysical Research Communications 451 (2014) 497–502

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# cNMP-AMs mimic and dissect bacterial nucleotidyl cyclase toxin effects

Ulrike Beckert [a,1], Manuel Grundmann [b,1], Sabine Wolter [a,1], Frank Schwede [c,1], Holger Rehmann [d], Volkhard Kaever [e], Evi Kostenis [b], Roland Seifert [a,*]

[a] Institute of Pharmacology, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany
[b] Institute of Pharmaceutical Biology, Nussallee 6, D-53115 Bonn, Germany
[c] Biolog Life Science Institute, Flughafendamm 9a, D-28199 Bremen, Germany
[d] Molecular Cancer Research, University Medical Center Utrecht, Universiteitsweg 100, NL-3584 Utrecht, The Netherlands
[e] Core Unit Metabolomics, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany

ARTICLE INFO

*Article history:*
Received 18 July 2014
Available online 7 August 2014

*Keywords:*
cNMP-AM
Prodrug
cUMP
cCMP
Dynamic mass distribution
Gene expression

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.

* Corresponding author. Address: Institute of Pharmacology, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany. Fax: +49 511 532 4081.
*E-mail addresses:* beckert-ulrike81@email.de (U. Beckert), grundmann@uni-bonn.de (M. Grundmann), wolter.sabine@mh-hannover.de (S. Wolter), fs@biolog.de (F. Schwede), h.rehmann@UMCutrecht.nl (H. Rehmann), kaever.volkhard@mh-hannover.de (V. Kaever), kostenis@uni-bonn.de (E. Kostenis), seifert.roland@mh-hannover.de (R. Seifert).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.07.134
0006-291X/

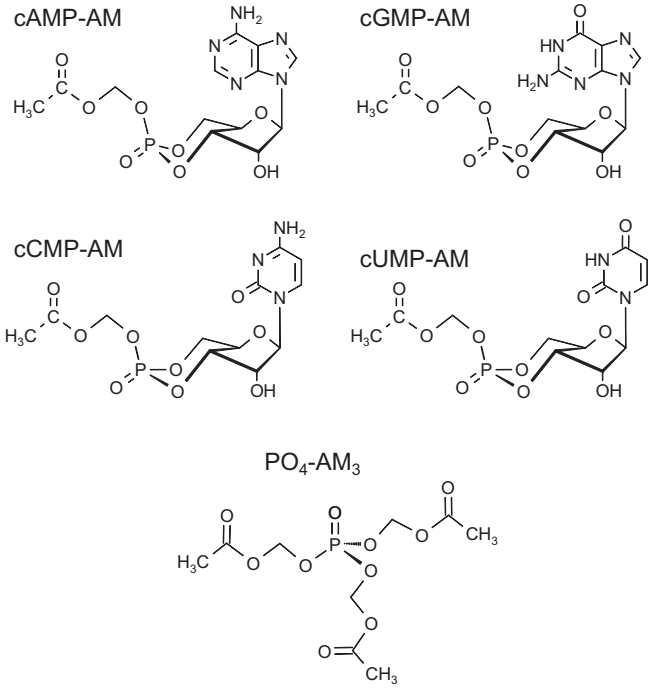


**Fig. 1.** Structures of cNMP-AMs and $PO_4$-$AM_3$. The AM group neutralizes the negative charge of the cNMP phosphate group. Accordingly, AM compounds can penetrate the plasma membrane. In the cytosol, esterases cleave the AM compounds, releasing the free cNMP and phosphate, respectively. Please note 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) $\lambda_{max}$ 270 nm ($\varepsilon$ = 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) $\lambda_{max}$ 260 nm ($\varepsilon$ = 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