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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-α_1 and pRC/CMV-β_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 α_1 and β_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 α_1 subunit (Sigma, Steinheim, Germany) and the β_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