FEBS Letters 588 (2014) 3469–3474

journal homepage: www.FEBSLetters.org

PDE7A1 hydrolyzes cCMP

Maike Monzel [a], Maike Kuhn [a,1], Heike Bähre [a,b], Roland Seifert [a], Erich H. Schneider [a,*]

[a] Institute of Pharmacology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany
[b] Core Facility Mass Spectrometry and Metabolomics, Institute of Pharmacology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

ARTICLE INFO

Article history:
Received 13 June 2014
Revised 2 August 2014
Accepted 4 August 2014
Available online 13 August 2014

Edited by Peter Brzezinski

Keywords:
Phosphodiesterase
HPLC–MS
Cyclic nucleotides
Cyclic CMP
Second messenger
Enzyme kinetics

ABSTRACT

The degradation and biological role of the cyclic pyrimidine nucleotide cCMP is largely elusive. We investigated nucleoside 3′,5′-cyclic monophosphate (cNMP) specificity of six different recombinant phosphodiesterases (PDEs) by using a highly-sensitive HPLC–MS/MS detection method. PDE7A1 was the only enzyme that hydrolyzed significant amounts of cCMP. Enzyme kinetic studies using purified GST-tagged truncated PDE7A1 revealed a cCMP K_M value of 135 ± 19 μM. The V_{max} for cCMP hydrolysis reached 745 ± 27 nmol/(min mg), which is about 6-fold higher than the corresponding velocity for adenosine 3′,5′-cyclic monophosphate (cAMP) degradation. In summary, PDE7A is a high-speed and low-affinity PDE for cCMP.

1. Introduction

Physiological role and metabolism of the pyrimidine nucleotide 3′,5′-cCMP are still largely elusive. The existence of a cCMP-forming cytidylyl cyclase was proposed [1], but technical problems resulted in a false-positive detection of cCMP [2]. The lack of sensitive detection methods hampered research activity in this field for many years. With the advent of highly sensitive and selective mass spectrometry-based detection systems [3], it became possible to detect even very low concentrations of cyclic pyrimidine nucleotides in cells, tissues and enzymatically digested samples [4,5]. cCMP is synthesized by soluble adenylyl cyclase [4], by soluble guanylyl cyclase [6] and by the *Pseudomonas aeruginosa* exotoxin ExoY [7]. It occurs in numerous cell types like human embryonic kidney cell line (HEK-293) cells and rat B103 neuroblastoma cells and primary cells from the neuronal, mesenchymal or epithelial lineage [8]. The activity of hyperpolarization-activated cyclic nucleotide-gated cation channel (HCN) ion channels is modulated by cCMP [9]. Furthermore, cCMP-agarose binds protein kinase A (PKA) [10] and cCMP regulates both PKA and protein kinase G (PKG) in vitro [11]. cCMP causes guanosine 3′,5′-cyclic monophosphate (cGMP) kinase I activation and subsequent relaxation of murine aorta smooth muscle cells [12]. If cCMP is a second messenger like the purine nucleotides adenosine 3′,5′-cyclic monophosphate (cAMP) and cCMP, it needs inactivation mechanisms to switch off the intracellular signal. A so-called "cCMP-specific" phosphodiesterase (PDE) was claimed with K_M values in the millimolar range [13–16]. Moreover, a "multifunctional cCMP-PDE" was proposed with K_M values <200 μM and activity for both 3′,5′- and 2′3′-cNMPs [16–21]. However, the identity of these proteins is still elusive.

We studied nucleoside 3′,5′-cyclic monophosphate (cNMP) specificity of eight recombinant PDEs (PDE1B, 2A, 3A, 3B, 4B, 5A, 8A and 9A), but, despite their broad substrate specificity, none of these enzymes accepted cCMP as a substrate [5]. In this publication we identified PDE7A as a cCMP-degrading PDE.

Abbreviations: A549, human lung carcinoma cell line; AMP, adenosine 5′-monophosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; B103, rat neuroblastoma cells; CMP, cytidine 5′-monophosphate; cCMP, cytidine 3′,5′-cyclic monophosphate; CyaA, *Bordetella pertussis* adenylyl cyclase toxin; ExoY, *P. aeruginosa* exotoxin Y; GMP, guanosine 5′-monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; GST, glutathione-S-transferase protein tag; HCN, hyperpolarization-activated cyclic nucleotide-gated cation channel; HEK-293, human embryonic kidney cell line; HIS, hexahistidine tag; HPLC–MS/MS, high performance liquid chromatography tandem mass spectrometry; HuT 78, human lymphoblast cell line; IMP, inosine 5′-monophosphate; cIMP, inosine 3′,5′-cyclic monophosphate; MRP5, multidrug resistance-associated protein 5; cNMP, nucleoside 3′,5′-cyclic monophosphate; PDE, phosphodiesterase; PKA, protein kinase A; PKG, protein kinase G; Sf9, *Spodoptera frugiperda* insect cell line; TMP, thymidine 5′-monophosphate; cTMP, thymidine 3′,5′-cyclic monophosphate; UMP, uridine 5′-monophosphate; cUMP, uridine 3′,5′-cyclic monophosphate; U-937, human promonocytic cell line; XMP, xanthosine 5′-monophosphate; cXMP, xanthosine 3′,5′-cyclic monophosphate

* Corresponding author.
[1] Current affiliation: TWINCORE Center for Experimental and Clinical Infection Research, Feodor-Lynen-Str. 7, 30625 Hannover, Germany.

http://dx.doi.org/10.1016/j.febslet.2014.08.005
0014-5793/

2. Materials and methods

2.1. Enzymes and reagents

Recombinant truncated PDE7A1/2 (shared sequence of PDE7A1 and PDE7A2, starting at amino acid 121 of Gene bank #NM_002603) was obtained from *BPS Bioscience* (San Diego, CA, USA). The enzyme was N-terminally GST-tagged, had a purity of >44% and an activity of 221 pmol/min/μg. Full length N-terminally HIS-tagged PDE1A3, 6AB, 7A1, 10A1 and 11A1 (lysates from baculovirus-infected *Spodoptera frugiperda* insect cell line (Sf9) cells, 0.5 pmol/min/μl) and a control lysate from uninfected Sf9 cells were purchased from *sb drug discovery* (Glasgow, UK). Detailed information on the purity and quality of the different PDE preparations is provided in the Supplementary Methods (1.3). Calmodulin was obtained from Calbiochem (Merck, Darmstadt, Germany). The internal standard tenofovir was obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD, USA). The selective and competitive PDE7A inhibitor BRL-50481 (5-Nitro-2,N,N-trimethylbenzenesulfonamide) was obtained from *TOCRIS Bioscience* (Bristol, UK) and dissolved in DMSO to yield a 5 mM stock solution. Adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), thymidine 5′-monophosphate (TMP), uridine 5′-monophosphate (UMP), inosine 5′-monophosphate (IMP) and xanthosine 5′-monophosphate (XMP) were from Sigma–Aldrich (Steinheim, Germany). The cyclic 3′,5′-nucleotides cAMP, cCMP, cGMP, inosine 3′,5′-cyclic monophosphate (cIMP), thymidine 3′,5′-cyclic monophosphate (cTMP), uridine 3′,5′-cyclic monophosphate (cUMP) and xanthosine 3′,5′-cyclic monophosphate (cXMP) were purchased from Biolog Life Science Institute (Bremen, Germany). All other reagents were analytical grade and from standard suppliers.

2.2. Screening of enzymes with various cNMPs

Hydrolytic activity of various PDEs was determined in 1x PDE buffer (50 mM Tris–HCl, 1.7 mM EDTA, 8.3 mM $MgCl_2$). For analysis of PDE1A3 activity, 100 μM $CaCl_2$, 100 mM EGTA and 100 nM calmodulin were added. PDE1A3, PDE6AB, PDE7A1 and PDE11A1 were analyzed at a volume activity of 5 pmol/(min ml). PDE10A1 was added at a volume activity of 12.5 pmol/(min ml). GST-tagged and truncated PDE7A was used at a concentration of 0.95 μg/ml, corresponding to 210 pmol/(min ml). Cyclic nucleotides were added to yield a final concentration of 3 μM. Incubation was performed for 1–24 h at 30 °C. Enzyme was inactivated by 15 min incubation at 95 °C. Protein was precipitated by freezing and removed by centrifugation. The supernatant was diluted 1:5 with purified water and then additionally mixed with an equal volume of tenofovir solution (internal standard), yielding a final tenofovir concentration of 50 ng/mL. The samples were analyzed by high performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) as described in the Supplementary Methods.

2.3. Time course of PDE7A activity

Time course experiments were performed in PDE buffer + 0.05% (m/v) BSA at a cAMP- or cCMP-concentration of 3 μM. The purified GST-tagged and truncated PDE7A1/2 was added at a concentration of 0.95 μg/ml, corresponding to 210 pmol/(min ml). Crude PDE7A1-containing Sf9 cell lysate was added to yield a final activity of 5 U/ml. The samples were incubated at 30 °C under constant shaking. Aliquots were drawn at appropriate times, processed as described for the enzyme screening experiments (Section 2.2) and analyzed by HPLC–MS/MS as described in the Supplementary Methods. In all experiments, the samples were repeatedly subjected to short centrifugations (at least every 30 min) to avoid concentration changes by evaporation- and condensation processes.

2.4. Determination of cCMP K_M and V_{max} for the GST-tagged purified PDE7A1/2

Michaelis Menten kinetics was studied in PDE buffer + 0.05% (m/v) BSA at an enzyme concentration of 0.95 μg/ml, corresponding to 210 pmol/(min ml) in the presence of increasing concentrations of cyclic nucleotide. Enzyme-free control samples were run in parallel for each individual cyclic nucleotide concentration. After an incubation of 1 h at 30 °C, the samples were stopped, processed and analyzed by HPLC–MS/MS as described in Section 2.2 and in the Supplementary Methods. Only samples with <40% substrate hydrolysis were included into data analysis.

A rough estimation of the kinetics of PDE7A1/2-mediated cAMP hydrolysis was performed by incubating 50 nM, 75 nM, 100 nM and 250 nM of cAMP for 6 min at 30 °C in the presence of 0.06–0.24 μg/ml of enzyme. Only samples with <60% substrate degradation in at least one of the duplicates were included into analysis.

2.5. Determination of the effect of the competitive inhibitor BRL-50481 on PDE7A activity

The effect of the competitive inhibitor BRL-50481 on PDE7A1/2 activity was studied at concentrations between 10 nM and 100 μM in PDE buffer + 0.05% (m/v) BSA. Incubations were performed for 15 min (cAMP) or 4 h (cCMP) at 30 °C in the presence of 500 nM (cAMP) or 10 μM (cCMP) of cyclic nucleotide. The GST-tagged PDE7A1/2 was used at 0.48 μg/ml, corresponding to 105 pmol/(min ml) for cAMP hydrolysis. For cCMP experiments 0.95 μg/ml enzyme were used, corresponding to 210 pmol/(min ml). DMSO content of all samples was adjusted to 2%.

2.6. Quantitation of cyclic nucleotides (cNMPs) and nucleotide monophosphates (NMPs) by HPLC–MS/MS and data analysis

The concentrations of the cNMPs and NMPs were determined by HPLC–MS/MS, consisting of an UFLC HPLC system (Shimadzu, Kyoto, Japan) and the QTRAP5500™ triple quadrupole mass spectrometer (ABSciex, Framingham, MA, USA). A detailed description of HPLC configuration, eluent composition, *m/z* values, retention times and data analysis is provided in the Supplementary Methods (1.1 and 1.2).

2.7. Preparation of whole cell homogenates and Western blots

The preparation of whole cell homogenates and Western blotting for Suppl. Fig. 1 are described in detail in the Supplementary information (Suppl. Methods 1.4 and 1.5).

3. Results

3.1. Analysis of substrate specificity and identification of PDE7A1 as cCMP-hydrolyzing PDE

The substrate specificity of PDE1A3, 6AB, 7A1, 10A1 and 11A1 (HIS-tagged full-length enzymes) was characterized in crude Sf9 cell lysates. The PDEs were incubated in the presence of 3′,5′-cAMP, -cGMP, -cCMP, -cUMP, -cXMP, -cIMP and -cTMP (3 μM each) for 24 h. In case of <100 % hydrolysis, the velocity of the enzymatic reaction was calculated. When 100 % of the specific cNMP was hydrolyzed within 24 h, incubation was shortened until hydrolysis was <100%. Since the enzymes were not purified, a lysate from uninfected Sf9 cells was used as a negative control. The PDE-overexpressing Sf9