Naunyn-Schmiedeberg's Arch Pharmacol (2017) 390:269–280
DOI 10.1007/s00210-016-1328-1

ORIGINAL ARTICLE

cUMP hydrolysis by PDE3A

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Received: 1 July 2016 / Accepted: 30 November 2016 / Published online: 14 December 2016

Abstract As previously reported, the cardiac phosphodiesterase PDE3A hydrolyzes cUMP. Moreover, cUMP-degrading activity was detected in cow and dog hearts several decades ago. Our aim was to characterize the enzyme kinetic parameters of PDE3A-mediated cUMP hydrolysis and to investigate whether cUMP and cUMP-hydrolyzing PDEs are present in cardiomyocytes. PDE3A-mediated cUMP hydrolysis was characterized in time course, inhibitor, and Michaelis-Menten kinetics experiments. Intracellular cyclic nucleotide (cNMP) concentrations and the mRNAs of cUMP-degrading PDEs were quantitated in neonatal rat cardiomyocytes (NRCMs) and murine HL-1 cardiomyogenic cells. Moreover, we investigated cUMP degradation in HL-1 cell homogenates and intact cells. Educts (cNMPs) and products (NMPs) of the PDE reactions were detected by HPLC-coupled tandem mass spectrometry. PDE3A degraded cUMP (measurement of UMP formation) with a K_M value of ~143 μM and a V_{max} value of ~42 μmol/min/mg. PDE3A hydrolyzed cAMP with a K_M value of ~0.7 μM and a V_{max} of ~1.2 μmol/min/mg (determination of AMP formation). The PDE3 inhibitor milrinone inhibited cUMP hydrolysis (determination of UMP formation) by PDE3A (K_i = 57 nM). Significant amounts of cUMP as well as of PDE3A mRNA (in addition to PDE3B and PDE9A transcripts) were detected in HL-1 cells and NRCMs. Although HL-1 cell homogenates contain a milrinone-sensitive cUMP-hydrolyzing activity, intact HL-1 cells may use additional PDE3-independent mechanisms for cUMP disposal. PDE3A is a low-affinity and high-velocity PDE for cUMP. Future studies should investigate biological effects of cUMP in cardiomyocytes and the role of PDE3A in detoxifying high intracellular cUMP concentrations under pathophysiological conditions.

Keywords Cyclic UMP · Phosphodiesterase · PDE3A · Neonatal rat cardiomyocytes · HL-1 cells

Introduction

The cyclic purine nucleotides cAMP and cGMP are well-established intracellular signaling molecules and fulfill all criteria of classic second messengers (Seifert 2015). Although there was some initial interest in the cyclic pyrimidine nucleotides cUMP and cCMP, research in this field was severely hampered by the lack of sensitive and selective detection methods (Seifert et al. 2015). With the advent of highly sensitive and specific mass-spectrometry-based methods, however, detection and quantitation of low concentrations of cUMP and cCMP became possible even in complex cellular matrices (Seifert et al. 2015). HPLC-coupled tandem mass spectrometry (HPLC-MS/MS) has revealed that significant concentrations of cUMP and cCMP are present in mammalian cells (Hartwig et al. 2014) and are generated by soluble guanylyl cyclase (sGC) (Bähre et al. 2014) or soluble adenylyl cyclase (sAC) (Hasan et al. 2014). Bacterial toxins such as

Electronic supplementary material The online version of this article (doi:10.1007/s00210-016-1328-1) contains supplementary material, which is available to authorized users.

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CyaA from *Bordetella pertussis* (Göttle et al. 2010) and ExoY from *Pseudomonas aeruginosa* (Beckert et al. 2014) are capable of producing cCMP and cUMP. Infection with *P. aeruginosa* results in high cUMP production in the mouse model, suggesting a pathophysiological role of this cyclic nucleotide (Bähre et al. 2015). In fact, the protein kinases PKA and PKG are stimulated by high concentrations of cCMP or cUMP in vitro (Wolter et al. 2011), and cCMP activates HCN channels with low potency and as a partial agonist (Zong et al. 2012). Moreover, cCMP induces caspase-dependent apoptosis of mouse lymphoma cells (Wolter et al. 2015). These examples support the notion that cUMP and/or cCMP production by bacterial toxins may exert biological effects and damage host cells.

Thus, it is important to elucidate the inactivation mechanisms of cyclic pyrimidine nucleotides. Multidrug resistance proteins (MRPs) 4 and 5 export cCMP and/or cUMP (Laue et al. 2014). A screening of 13 recombinant phosphodiesterases (PDEs) revealed only one enzyme (PDE7A) that hydrolyzes cCMP, but three enzymes (PDE3A, 3B, and 9A) that degrade cUMP with significant activity (Reinecke et al. 2011; Monzel et al. 2014). Recently, our research group has characterized the enzyme kinetics of PDE7A-mediated cCMP hydrolysis, revealing that PDE7A1/2 is a low-affinity and high-velocity enzyme for cCMP (Monzel et al. 2014). In this paper, we report on the detailed characterization of PDE3A-mediated cUMP degradation. Moreover, we demonstrate the presence of significant amounts of cUMP in cardiomyocytes (neonatal rat cardiomyocytes and murine cardiomyogenic HL-1 cells) and show that cUMP hydrolysis in HL-1 homogenates is sensitive to milrinone. Our results strongly suggest that the cUMP-degrading PDE activity detected in cow and dog heart tissue 50 years ago (Hardman and Sutherland 1965) was at least partially caused by cardiac PDE3A.

Materials and methods

Enzymes, reagents, and buffers

Truncated (amino acids 484–1141) recombinant N-terminally GST-tagged human PDE3A (lot #130312-G1; GenBank accession no. NM_000921) with a purity of ≥25% (http://bpsbioscience.com/pde3a-484-end-60030) and a specific activity of 1100 pmol/min/μg was obtained from BPS Bioscience (San Diego, CA, USA). 3′,5′-cAMP, 3′,5′-cCMP, 3′,5′-cGMP, and 3′,5′-cUMP were provided by Sigma Aldrich (Steinheim, Germany). The acetoxymethyl (AM) ester of cUMP, cUMP-AM, was obtained from Biolog Life Science Institute (Bremen, Germany). The PDE3-selective inhibitor milrinone (lot #23922111; LKT-M3344) was purchased from Biomol GmbH (Hamburg, Germany) and dissolved in DMSO to yield a 200-mM stock solution. The internal standard tenofovir was obtained from the National Institute of Health (NIH) AIDS Research and Reference Reagent Program (Germantown, MD, USA). Claycomb medium was purchased from Sigma Aldrich and supplemented with 0.1 mM of norepinephrine by adding an appropriate volume of a 10-mM norepinephrine stock solution containing 30 mM of ascorbic acid. Additionally, a mixture of penicillin, streptomycin, and L-glutamine as well as 10% fetal bovine serum (FBS) were added as previously described (Claycomb et al. 1998). All PDE reactions were performed in 1× PDE buffer containing 50 mM of Tris–HCl (pH 7.5), 8.3 mM of magnesium chloride, and 1.7 mM of EDTA. For the experiments with recombinant enzyme, the 1× PDE buffer was additionally supplemented with 0.05% BSA. All other reagents were analytical grade and from standard suppliers.

Time course of PDE3A activity for cAMP and cUMP

Recombinant GST-tagged PDE3A was added to a solution containing 3 μM of cAMP or cUMP in 1× PDE buffer supplemented with 0.05% of BSA, yielding a final volume of 300 μl and a final enzyme concentration of 0.2 μg/ml. All samples were run in duplicates. The total incubation time was 90 min at 30 °C under constant shaking at 300 rpm (Eppendorf Thermomixer® comfort). As 0 min value, the negative control without enzyme was used. During the incubation time, aliquots of 40 μl were drawn at defined times and instantly heated to 95 °C for 15 min to inactivate PDE3A and precipitate protein. After freezing the samples at −20 °C overnight, they were thawed and centrifuged (15 min, 20,800×*g*, 4 °C) to remove precipitated protein. Ten microliters of the supernatant was diluted 1:4 with purified water. An equal volume of tenofovir solution (100 ng/ml) was added to yield a final tenofovir concentration of 50 ng/ml and a final volume of 80 μl. Degradation of the substrate (cAMP or cUMP) as well as accumulation of the product (AMP or UMP) was determined by high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) as previously described (Monzel et al. 2014).

Michaelis-Menten analysis of PDE3A-mediated cUMP and cAMP degradation

For the determination of K_M and V_{max} values of PDE3A-mediated cAMP and cUMP hydrolysis, substrate concentrations between 0.1 and 6 μM (cAMP) and between 1 μM and 1.5 mM (cUMP) were used in 1× PDE buffer supplemented with 0.05% of BSA. The reaction was started by adding PDE3A to yield a final concentration of 0.1 μg/ml and a final sample volume of 50 μl. Negative controls without PDE were run in parallel for each substrate concentration. All samples were run in duplicates and incubated for 15 min (cUMP) or 2 min (cAMP) at 30 °C under constant shaking (300 rpm). After that, 40 μl aliquots were drawn, heat-inactivated, and

frozen overnight as described in the section about the time course experiments.

In the next step, the samples were thawed and centrifuged (15 min, 20,800×*g*, 4 °C) to remove precipitated protein. In case of samples with less than 10 μM of cNMPs, 10 μl of the supernatant was diluted 1:4 with purified water. For cNMP concentrations of more than 10 μM and less than 0.5 mM, the supernatant was diluted 1:200 (1:50, followed by 1:4) with purified water. Substrate concentrations higher than 0.5 mM were diluted 1:400 (1:100, followed by 1:4). Forty microliters of these dilutions were mixed 1:1 with tenofovir solution (100 ng/ml) to yield a final tenofovir concentration of 50 ng/ml and a final volume of 80 μl. The HPLC-MS/MS measurement was performed as previously described (Monzel et al. 2014).

Inhibition of PDE3A-mediated cUMP and cAMP hydrolysis by milrinone

The reaction was started by adding PDE3A to 1× PDE buffer containing appropriate milrinone concentrations (0–10 μM), 0.05% DMSO, and cAMP (1 μM) or cUMP (3 μM). The final PDE3A concentration was 0.2 μg/ml (cUMP samples) or 0.1 μg/ml (cAMP samples). The sample volume was 50 μl and all samples were run in duplicate. After an incubation for 10 min (cUMP) or 15 min (cAMP) at 30 °C, the samples were stopped, processed, and analyzed as described in the section about the time course experiments. The HPLC-MS/MS measurement was performed as previously described (Monzel et al. 2014).

Cell culture

The mouse cardiomyogenic cell line HL-1 was cultured in supplemented Claycomb medium as previously described (Claycomb et al. 1998). The media was changed every 24 to 48 h. Prior to seeding the cells, cell culture flasks and dishes were coated with a dilution of 0.005 mg/ml fibronectin in 0.02% gelatin for at least 60 min at 37 °C. Neonatal rat cardiomyocytes (NRCM) were isolated as described previously (Hilfiker-Kleiner et al. 2004) and cultured in 60 × 15 mm cell culture dishes coated with 1% gelatin at a density of 1.2–1.5 × 10^6 cells/dish in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Medium 199, 5% fetal calf serum (FCS), and 10% horse serum. 3T3-L1-MBX preadipocyte cells were obtained from LGC Standards/ATCC (Wesel, Germany) and cultured in DMEM supplemented with 10% neonatal calf serum (NCS), penicillin, streptomycin, and L-glutamine.

Preparation of cell homogenates

Both HL-1 cells and 3T3-L1-MBX cells were cultured until 70–80% confluence in 175 cm^2 cell culture flasks as described in the section about cell culture conditions. Cells were trypsinized, pelleted at 300×*g*, and washed three times in PBS (1×). After that, they were re-suspended in BSA-free 1× PDE buffer supplemented with a protease-inhibitor mixture consisting of benzamidine (5 mM), phenylmethylsulfonylfluoride (PMSF; 200 μM), and leupeptin (57.4 μM). After an incubation of 10 min, cells were either homogenized by applying ~50 strokes in a 5-ml Dounce homogenizer or by ~30 s of sonification (Branson Sonifier Analog 250). All steps were performed on ice. After one freeze-thaw cycle, protein concentrations were determined using the Pierce® BCA Protein Assay from Thermo Scientific (Rockford, IL, USA) according to the manufacturer's instructions. The cell homogenates were stored at −80 °C.

Basal cUMP content (HL-1 cells or NRCMs) and PDE3-mediated cUMP hydrolysis in cell homogenates (HL-1 cells)

The experiments with HL-1 cell homogenates (prepared as described in the preceding section) were run in duplicate in BSA-free PDE buffer and at a final protein concentration of 250 μg/ml. The samples contained 3 μM of substrate (cAMP or cUMP) and either 10 μM of milrinone or 0.005% of DMSO (negative control). Aliquots were drawn right at the beginning (0 min) and after 6 h of incubation at 30 °C (constant shaking at 300 rpm). The tubes were centrifuged approximately every 30 min to minimize the error caused by condensation of liquid in the lid. Afterwards, samples were stopped, processed, and analyzed as described in the section about the time course experiments.

For determination of intracellular cNMP concentrations, HL-1 cells were grown to 70–80% confluence in six-well plates. The medium was removed and 300 μl of ice-cold extraction solution (acetonitrile/methanol/water; 2:2:1; *v*/*v*/*v*) containing 25 ng/ml tenofovir as internal standard was added. The cells were rigorously scraped off and the suspension was transferred to 2-ml reaction tubes. The wells were washed twice with 400 μl of ice-cold extraction solution and the suspension from both washing steps was also transferred to the 2-ml reaction tube, yielding a final volume of 1.1 ml. NRCMs were isolated from neonatal rat brains and seeded in 6-cm dishes. On the fourth or fifth day of cultivation, the medium was removed and 500 μl of ice-cold extraction solution without tenofovir plus 300 μl of extraction solution containing 25 ng/ml tenofovir were added. The following steps were performed as described for HL-1 cells, but with 500 μl of extraction solution for washing, yielding a final volume of 1.8 ml. The suspensions from the extraction of HL-1 cells or NRCMs were heated to 95 °C for 15 min and then stored at −20 °C overnight. After thawing and centrifuging for 10 min at 20,800×*g*, the supernatant was transferred into a new tube and evaporated completely at 40 °C under a constant nitrogen

stream. The residue was dissolved in 150 μl of water. The cNMP measurement was performed as previously described (Hasan et al., 2014). The cNMP content was normalized to the protein concentration of each sample, which was determined using the Pierce® BCA Protein Assay from Thermo Scientific (Rockford, IL, USA) according to the manufacturer's instructions.

Effect of milrinone on cNMP concentrations in intact HL-1 cells after pre-incubation with cUMP-AM

HL-1 cells were grown to 70–80% confluence in six-well plates. On the day of the experiment, the cells were pre-incubated with 10 μM of cUMP-AM or with 0.1% DMSO (control) for 5 min. Then the medium was aspirated and the cells were washed with PBS once. After that, supplemented Claycomb medium containing either 100 μM of milrinone or 0.5% DMSO (control) was added to the cells. After incubation for predefined times (0, 30, 60, and 120 min) at 37 °C and 5% CO_2, the medium was removed and 300 μl of ice-cold extraction solution (acetonitrile/methanol/water; 2:2:1; *v*/*v*/*v*) containing 25 ng/ml tenofovir as internal standard were added. The samples were processed as described in the preceding section.

Quantitative real-time PCR (qPCR)

Preparation of RNA from HL-1 cells, nRCMs, and 3T3-L1-MBX cells was performed using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The RNA was reverse-transcribed into cDNA and analyzed using TaqMan® probes for the expression of mouse (mActb; Mm00607939_s1; ThermoFisher Scientific, Frankfurt/Main, Germany) and rat (rActb; Rn00667869_m1) β-actin as well as mouse and rat PDE3A (mouse—Mm00479581_m1; rat—Rn00569192_m1), PDE3B (mouse—Mm00691635_m1; rat—Rn00568191_m1), and PDE9A (mouse—Mm00501039_m1; rat—Rn00593577_m1). Reactions were performed with TaqMan® Gene Expression Master Mix (Applied Biosystems™, Frankfurt, Germany). The mRNA expression levels of the genes were compared after calculating the ΔC_T values:

$$\Delta C_T = C_{T\ [\text{target gene}]} - C_{T\ [\text{m or rActb}]}$$

Western blots

After diluting the samples with 5× Lane Marker Reducing Sample Buffer™ (#39000; Thermo Scientific, Bremen, Germany), they were heated to 96 °C for 10 min. Then, the samples were loaded on an SDS polyacrylamide gel (5% stacking and 7.5% resolution gel), which was run at 25 mA for 10 min and at 35 mA for 60 min. The protein was transferred to a PVDF membrane by semi-dry blotting (2 mA/cm^2, 90 min). After that, the membrane was blocked in Roti® Block protein-free blocking solution (Roth, Karlsruhe, Germany) for 60 min at ambient temperature and then incubated overnight at 4 °C with a rabbit monoclonal antibody against PDE3A (EPR11601, cat. #ab169534; Abcam, Cambridge, MA, USA) at a 1:1000 dilution in Roti® Block. On the next day, the membrane was washed 3 × 7 min with 1× TBST and then incubated for 120 min at ambient temperature with an HRP-linked goat-anti-rabbit secondary antibody (#7074S; Cell Signaling Technology, NEB Frankfurt, Germany), diluted 1:3000 in Roti® Block. Finally, the blot was developed by applying a 1:1 mixture of Western Sure Luminol Enhancer Solution and Western Sure Stable Peroxidase Solution (LI-COR Biotechnology, Bad Homburg, Germany). Luminescence was detected using a C-Digit® Blot scanner (LI-COR Biotechnology).

Data analysis and statistics

HPLC-MS/MS-data were analyzed with the Analyst Software (ABSciex; Framingham, USA), and the results were exported to Microsoft Excel® (Microsoft; Redmond, USA). The area of every cNMP and/or NMP chromatography peak was determined for the samples as well as for appropriate standard curves containing AMP, cAMP, UMP, and cUMP (0, 0.24, 0.49, 0.98, 1.95, 3.9, 7.8, 15.6, 31.3, 62.5, and 125 pmol) or all four cNMPs and their 5′-NMP hydrolysis products (0.0262, 0.0655, 0.164, 0.410, 1.024, 2.56, 6.4, 16, 40, 100, and 250 pmol). Using GraphPad Prism 6® (San Diego, CA, USA), the individual analyte amounts were calculated from the peak areas. In order to minimize matrix effects, the peak area of the analyte was related to the peak area of the internal standard tenofovir in studies with cell homogenates. For the analysis of enzyme kinetics data, tenofovir was not included. Data plots and calculations of K_M, V_{max}, and IC_{50} values were performed with GraphPad Prism 6® (San Diego, USA).

Results

Time course of PDE3A activity

In order to assess stability and activity of the recombinant PDE3A used in our experiments, we determined the time course of PDE3A-mediated cAMP and cUMP hydrolysis. cAMP (3 μM) was fully hydrolyzed within 20 min (Fig. 1a) at a constant velocity of about 0.86 μmol/min/mg (calculated from the first 10 min of the curve) (Fig. 1a, c). By contrast, about 60 min were required for complete hydrolysis of 3 μM of cUMP. Interestingly, the initial velocity of cUMP

hydrolysis (0.91 μmol/min/mg) was comparable to the initial cAMP degradation rate (Fig. 1b, c). After 10 min, however, cUMP hydrolysis slows down significantly (Fig. 1b).

Michaelis-Menten kinetics of PDE3A for cUMP and cAMP

For the PDE3A/cAMP Michaelis-Menten kinetics, 0.1–6 μM of substrate was applied (enzyme concentration 0.1 μg/ml). Calculation of K_M and V_{max} from the cAMP hydrolysis data (Fig. 2a, Table 1) yielded 0.26 μM and 1.30 μmol/min/mg, respectively (n = 2 in duplicates). The K_M value of AMP formation (Fig. 2b, Table 1) was 0.71 μM with a V_{max} of 1.15 μmol/min/mg (n = 2 in duplicates). The Michaelis-Menten kinetics of PDE3A-mediated cUMP hydrolysis was determined at substrate concentrations between 1 μM and 1.5 mM (enzyme concentration 0.1 μg/ml). Hydrolysis of cUMP (Fig. 2c) occurred with a K_M value of 98.8 μM and a V_{max} of 37.6 μmol/min/mg. The UMP accumulation data (Fig. 2d) yielded a K_M value of 142.9 μM and a V_{max} value of 42.0 μmol/min/mg (n = 4 in duplicates). These results are listed in Table 1. The complete datasets for cUMP hydrolysis and UMP accumulation in the Michaelis-Menten kinetics experiments are shown in Supplementary Tables 1 and 2.

Inhibition of PDE3A-mediated cUMP and cAMP hydrolysis by the PDE3-selective inhibitor milrinone

Milrinone, a selective inhibitor of PDE3, was used at concentrations between 1 nM and 100 μM to inhibit PDE3A-mediated hydrolysis of cUMP (3 μM) and cAMP (1 μM). As expected, milrinone inhibited cAMP degradation by PDE3A (Fig. 3a), yielding IC_{50} values of 899 nM (cAMP hydrolysis) and 1075 nM (AMP formation) (Table 1). Milrinone also reduced both PDE3A-mediated cUMP hydrolysis and UMP accumulation (Fig. 3b) with IC_{50} values of 122 ± 99.6 nM and 57.5 ± 37.3 nM, respectively (n = 3, means ± SEM). The K_i values were calculated by applying the Cheng-Prusoff equation (Cheng and Prusoff 1973), using the corresponding K_M values of the substrates (see preceding section and Table 1). For inhibition of PDE3A-mediated cAMP hydrolysis, the K_i values of milrinone were 186 nM (cAMP hydrolysis) and 446 nM (AMP formation). Hydrolysis of cUMP was inhibited by milrinone with a K_i value of 56 nM (UMP formation) and 118 nM (cUMP hydrolysis) (Table 1).

Basal cNMP levels in HL-1 cells and neonatal rat cardiomyocytes

PDE3A is expressed in heart (Omori and Kotera 2007) and the presence of a cUMP-hydrolyzing activity has been previously reported in cow and dog hearts (Hardman and Sutherland 1965). Thus, we chose HL-1 cardiomyogenic cells and neonatal rat cardiomyocytes (NRCMs) as model cell systems to investigate cUMP metabolism in these cells. HPLC-MS/MS analysis revealed the presence of all four analyzed cNMPs in both cell systems. The most abundant cNMP was cAMP with a concentration of 36.3 ± 10.7 pmol/mg in HL-1 cells and 200.8 ± 81.8 pmol/mg in NRCMs (n = 4, means ± SD) (Figs. 4a and 4b). The cUMP concentration in HL-1 cells was 3.1 ± 2.0 pmol/mg and was comparable to that of the established second messenger cGMP that amounted to 2.6 ± 1.0 pmol/mg (n = 4, means ± SD). Similarly, in NRCMs, the cGMP concentration was 10.8 ± 2.8 pmol/mg, while cUMP occurred at a concentration of 9.0 ± 2.4 pmol/mg (n = 4, means ± SD). In both cell types, the least abundant cNMP was cCMP (HL-1 = 1.6 ± 1.0 pmol/mg; NRCMs = 4.8 ± 1.4 pmol/mg; means ± SD from n = 4). The original datasets for all experiments are shown in Supplementary Table 3.

Expression of cUMP-degrading PDEs in HL-1 cells and neonatal rat cardiomyocytes

Next, we addressed the question which of the previously described (Reinecke et al. 2011) cUMP-hydrolyzing PDEs are expressed in cardiomyocytes. We isolated RNA from HL-1 cells and NRCMs and analyzed transcription of PDE3A, PDE3B, and PDE9A genes (Fig 5). The mRNA for all three cUMP-degrading enzymes was present in HL-1 cells (n = 3-4 independent RNA isolations, means ± SD) with ΔC_T values of 10.94 ± 1.36 (PDE3A), 7.96 ± 1.17 (PDE3B), and 13.35 ± 1.49 (PDE9A). Moreover, these mRNAs were detected in NRCMs (n = 2 independent RNA isolations, means ± SD) with ΔC_T values of 6.70 ± 0.31 (PDE3A), 7.16 ± 0.31 (PDE3B), and 14.38 ± 0.22 (PDE9A). According to the literature, 3T3-L1 fibroblasts show low PDE3B expression, which is only increased upon differentiation to adipocytes (Taira et al. 1993; Niiya et al. 2001). Thus, RNA from 3T3-L1-MBX fibroblasts (n = 2-3 independent RNA isolations) was used as a negative control for PDE3B, showing an approximate ΔC_T value of 16.5. The ΔC_T values for PDE3A (18.3) and PDE9A (18.5) suggest that 3T3-L1 cells are also suited as negative controls for PDE3A and 9A. All ΔC_T values were calculated using mActb as housekeeping gene.

We also tried to detect PDE3A on the protein level by Western blotting with a monoclonal rabbit anti-PDE3A antibody. In Hl-1 cells, the antibody stained two bands around 130 kDa (Supplementary Fig. 1A and B, red boxes) that might correspond to PDE3A isoforms. A 118-kDa and a 136-kDa PDE3A isoform have been previously reported for human myocardium (Wechsler et al. 2002). However, the antibody also stained numerous bands below 100 kDa, indicating a lack of selectivity. Moreover, a band close to 130 kDa was also

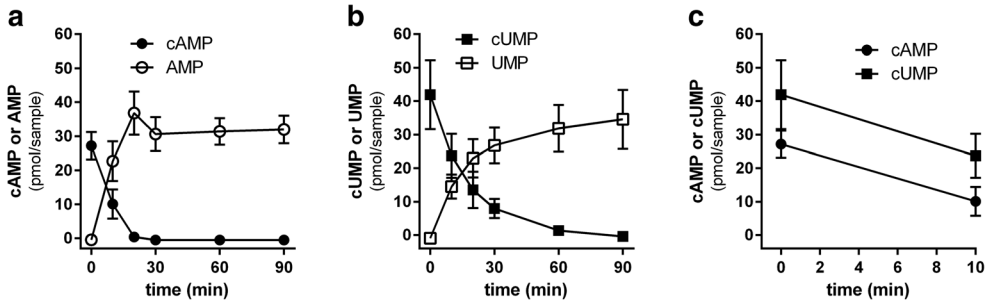

Fig. 1 Time course of PDE3A-mediated hydrolysis of 3′,5′-cAMP and 3′,5′-cUMP. **a** Degradation of cAMP (3 μM) and formation of AMP by purified GST-tagged PDE3A; **b** degradation of cUMP (3 μM) and formation of UMP by purified GST-tagged PDE3A; **c** comparison of the initial linear parts of the curves for PDE3A-mediated cAMP and cUMP hydrolysis; Data are means ± SD from $n = 3$ independent experiments performed in duplicates

detected in cell homogenates of HepG2 liver carcinoma cells, U937 promonocytes, and 3T3-L1 pre-adipocytes.

A clear staining between 100 and 130 kDa was observed with the recombinant enzyme preparation used for the PDE3A enzyme kinetics experiments in this paper (Supplementary Fig. 1A and B, red boxes). According to the manufacturer's specifications, the enzyme is truncated and bears an N-terminal GST tag, resulting in a net molecular mass of 103 kDa. In addition to the high molecular mass signal, however, the antibody also produced strong staining between 70 and 100 kDa in the recombinant protein sample (Supplementary Fig. 1A, lanes 2–4). It is unclear if this staining is due to PDE3A degradation products or caused by a totally different protein that coincidentally cross-reacts with the antibody. According to the manufacturer's datasheet, a Coomassie-stained SDS-PAGE of this preparation yielded numerous bands below 100 kDa and purity was given as ≥25%, which supports the notion that the preparation contains major impurities.

Inhibition of cUMP hydrolysis in HL-1 cell homogenates by milrinone

In the next step, we addressed the question whether the PDE3 isoforms constitutively expressed in HL-1 cardiomyogenic cells are capable of hydrolyzing cUMP. This question is specifically interesting because more than 50 years ago, a cUMP-hydrolyzing PDE activity was reported in beef and dog heart

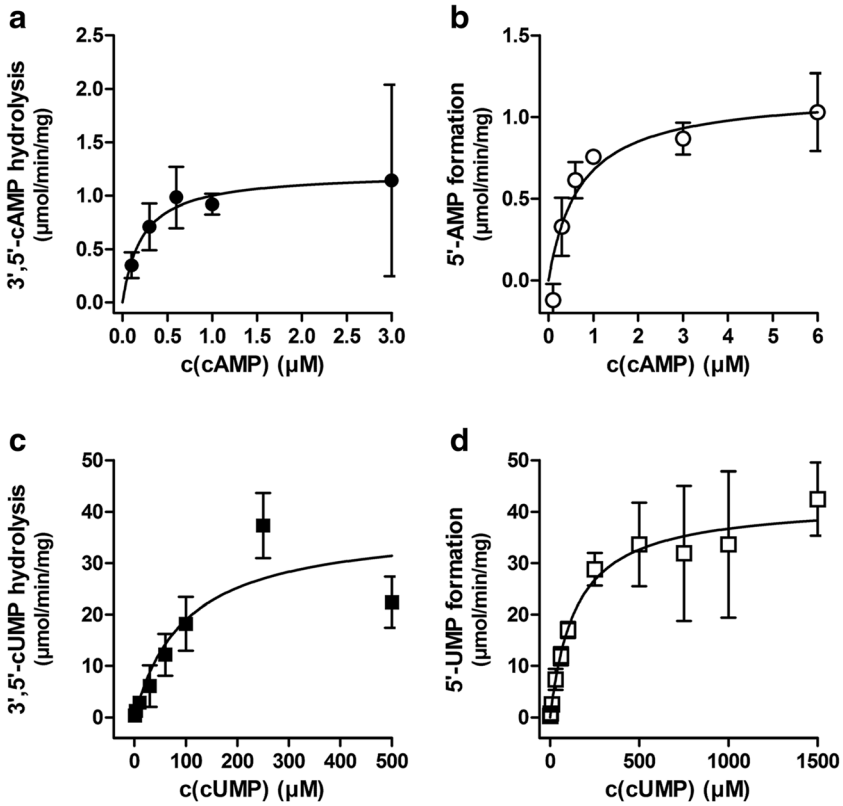

Fig. 2 Michaelis-Menten kinetics of PDE3A-mediated cNMP hydrolysis. Velocity of PDE3A-mediated **a** cAMP degradation and **b** AMP formation in the presence of increasing cAMP concentrations; velocity of PDE3A-mediated **c** cUMP degradation and **d** UMP formation in the presence of increasing concentrations of cUMP. Data are means ± SD from $n = 4$ (**c**, **d**) or $n = 2$ (**a**, **b**) independent experiments in duplicates. The mean K_M and V_{max} values are listed in Table 1. Moreover, the complete datasets for the cUMP Michaelis-Menten kinetics are shown in Supplementary Table 1 (UMP accumulation) and Supplementary Table 2 (cUMP hydrolysis)

Table 1. Characterization of GST-tagged PDE3A in terms of cUMP and cAMP degradation and UMP and AMP formation (data are means ± SEM, graphs are shown in Figs. 2 and 3)

Parameter	UMP formation	cUMP hydrolysis	AMP formation	cAMP hydrolysis
V_{max} [μmol/(min mg)]	42.0 ± 2.8 (n = 4)	37.6 ± 5.2 (n = 4)	1.15 (1.27, 1.03)[a]	1.30 (1.85, 0.76)[a]
K_M (μM)	142.9 ± 36.1 (n = 4)	98.8 ± 36.4 (n = 4)	0.71 (0.67, 0.75)[a]	0.26 (0.41, 0.11)[a]
IC_{50} (milrinone) (nM)	57.5 ± 37.3[b] (n = 3)	122 ± 99.6[b] (n = 3)	1075 (925, 1224)[a]	899 (1099, 699)[a]
K_i (milrinone) (nM)[c] (calculated from IC_{50})	56	118	446[d]	186[d]

[a] For experiments with n = 2, the results from the individual experiments were used to calculate the mean value. The individual results are given below in brackets

[b] The logarithmic error from the GraphPad prism output was converted to the linear error given in the table by multiplying the log error with the non-logarithmic mean value and with ln10

[c] Calculated with the Cheng-Prusoff equation by using the corresponding K_M values

[d] These K_i values may be overestimated due to substrate depletion (as explained in the discussion of this article)

tissue (Hardman and Sutherland 1965). We incubated HL-1 cell homogenates with 3 μM of cUMP for a total time of 6 h in the presence and absence of milrinone and analyzed the cNMP content by HPLC-MS/MS (n = 4 homogenates, tested in duplicates). HL-1 cell homogenate hydrolyzed a small but significant amount of cUMP (~13%) within 6 h, which was significantly inhibited by milrinone. Even after 6 h, the initially added cUMP concentration was only reduced by ~3%, when the PDE3 inhibitor was present (Fig. 6). By contrast, cAMP was completely degraded by the HL-1 cell homogenate during the 6-h incubation time. This was not prevented by milrinone (data not shown), probably due to the presence of other cAMP-hydrolyzing but milrinone-insensitive PDEs.

Inhibition of cUMP hydrolysis by milrinone in intact HL-1 cells

Next, we investigated whether milrinone inhibits cUMP hydrolysis in living HL-1 cells. The cells were incubated for 5 min with 10 μM of cUMP-AM (or DMSO as solvent control). Then, the cUMP-AM-containing incubation medium was removed, the cells were washed once and then incubated for 120 min in the presence of 100 μM of milrinone or solvent (DMSO, negative control). The time course of intracellular cUMP concentrations was followed by HPLC-MS/MS, yielding the data depicted in Fig. 7. Since no difference between milrinone-containing and milrinone-free samples is to be expected at t = 0 min, both sets of samples were merged to compare the average initial cUMP concentrations. At t = 0 min, the cUMP-AM pre-treated samples had a cUMP content of 41.1 ± 34.1 pmol/mg (n = 8, mean ± SD) (Fig. 7a), while the DMSO controls contained only 3.7 ± 0.4 pmol/mg of cUMP (n = 6, mean ± SD) (Fig. 7c). This indicates that cUMP-AM pre-incubation significantly increased intracellular cUMP ($p < 0.05$, unpaired two-tailed t test).

Intracellular cUMP concentrations dramatically decreased within 30 min in all cUMP-AM-pre-loaded samples (Fig. 7a, b). Since the initial cUMP concentration after pre-incubation

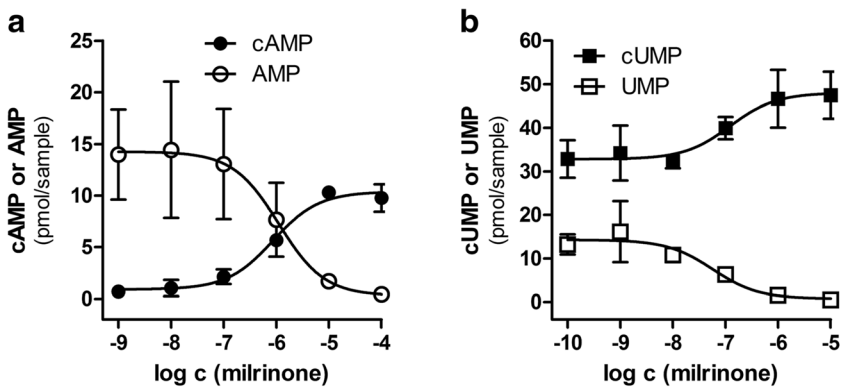

Fig. 3 Inhibition of PDE3A-mediated cAMP and cUMP hydrolysis by milrinone. Comparison of **a** degradation of 1 μM of cAMP (*filled circles*) and formation of AMP (*open circles*) as well as **b** degradation of 3 μM of cUMP (*filled squares*) and formation of UMP (*open squares*) by purified GST-tagged PDE3A in the presence of increasing milrinone concentrations. Data are means ± SD from n = 2 (cAMP) or n = 3 (cUMP) independent experiments performed in duplicates. The mean K_i values for inhibition of cUMP or cAMP degradation are listed in Table 1

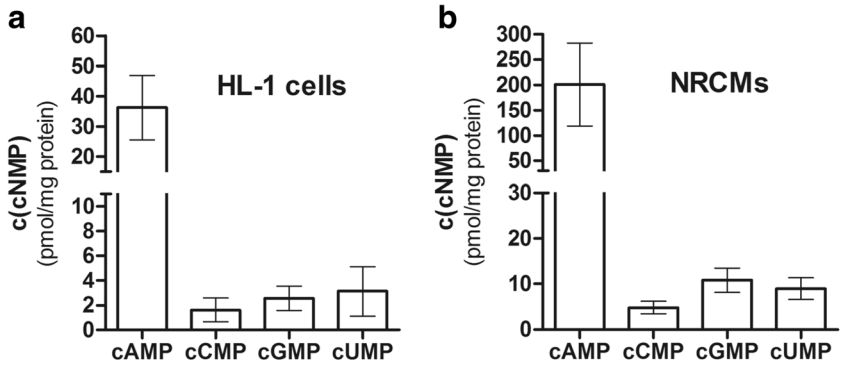

Fig. 4 Basal cNMP concentrations in HL-1 cardiomyogenic cells (**a**) and NRCMs (**b**), related to the protein amounts determined by the BCA method. Data are means ± SD from $n = 4$ independent experiments with 2–4 replicates. The mean values of the individual experiments are given in the text of the "Results" section. The complete datasets are given in Supplementary Table 3.

with cUMP-AM shows high inter-experimental variability (error bar at $t = 0$ min in Fig. 7a), all values were normalized to the cUMP concentration at $t = 0$ min (Fig. 7b). The DMSO controls were transformed the same way (Fig. 7c vs. d). The relative cUMP concentrations after 30, 60, and 120 min were significantly reduced as compared to $t = 0$ min in both the milrinone-containing ($p < 0.001$) and the milrinone-free ($p < 0.01$) samples (one-way ANOVA followed by Dunnett's multiple comparison test). After 120 min, the cUMP concentrations had stabilized at 10.5 ± 4.2 pmol/mg (with milrinone) and at 9.2 ± 4.5 pmol/mg (without milrinone) in the cUMP-AM-pre-incubated samples. In the solvent controls, the 120-min values were at 4.3 ± 2.2 pmol/mg (with milrinone) and at 3.9 ± 2.6 pmol/sample (without milrinone).

In addition to cUMP, we also quantified cAMP, cCMP, and cGMP in all samples (data not shown). Pre-incubation of the cells with cUMP-AM had no significant effect on cAMP, cGMP, and cCMP contents, and milrinone caused only a slight non-significant increase of intracellular cAMP (data not shown).

Discussion

A detailed enzyme kinetics analysis of PDE3A-mediated cUMP hydrolysis was provided. The initial hydrolysis rate in time course experiments was comparable for 3 μM of cAMP or cUMP. After 20 min, however, the reaction speed in the cUMP samples declined, while cAMP hydrolysis continued at a constant rate. This may be due to largely different K_M values. PDE3A is saturated by 3 μM of cAMP and therefore works at maximum speed throughout the experiment. By contrast, due to a higher cUMP K_M value, the enzyme is not saturated by 3 μM of cUMP and substrate depletion quickly causes a reduction of cUMP hydrolysis rate. Alternatively, the cUMP degradation could be slowed down by product inhibition or limited enzyme stability. The Michaelis-Menten kinetics data, however, support the notion that PDE3A shows a much lower affinity for cUMP than for cAMP. An about 200-fold higher K_M value and a 30-fold higher maximum reaction speed (V_{max}) of PDE3A for cUMP as compared to cAMP (product accumulation data) indicate that PDE3A is a low-affinity and high-

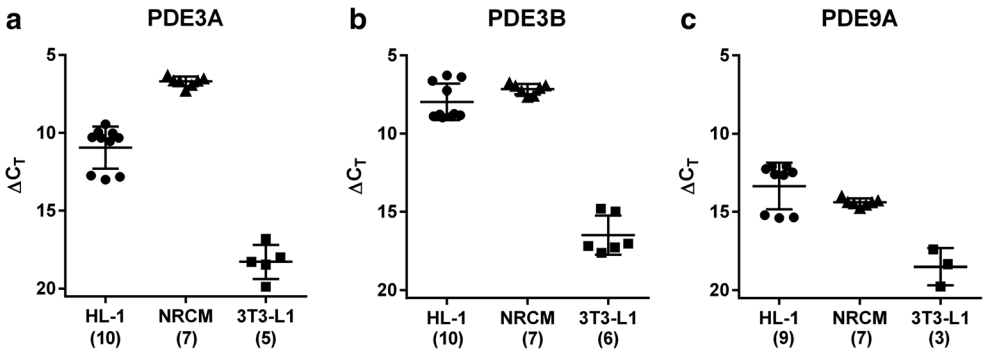

Fig. 5 Analysis of PDE3A, PDE3B, and PDE9A gene expression in HL-1 murine cardiomyogenic cells ($n = 3$-4 independent RNA isolations), NRCM ($n = 2$ independent RNA isolations), and murine 3T3-L1-MBX ($n = 2$-3 independent RNA isolations) pre-adipocytes. **a** PDE3A, **b** PDE3B, **c** PDE9A. Data were analyzed using the ΔC_T method with actin as a housekeeping gene. Please note the inverted relationship between expression level and ΔC_T value: a lower ΔC_T signifies a higher amount of transcript. The data points represent the individual samples from 2–4 independent RNA isolations. The number of qPCR samples (measured in duplicates) for each cell type and PDE is given in round parentheses below the x-axis

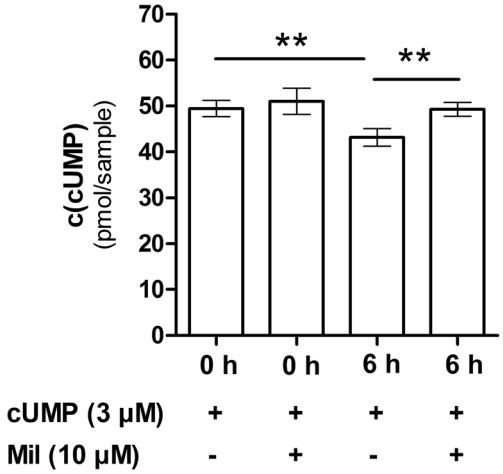

Fig. 6 Milrinone-sensitive cUMP-degrading activity in HL-1 cell homogenates. Depicted are cUMP concentrations in HL-1 cell homogenate samples in the beginning and at the end of a 6-h incubation period with 3 µM of cUMP. Milrinone (10 µM)-containing samples were compared with negative controls containing solvent (DMSO). Data are means ± SD from $n = 4$ different HL-1 cell homogenates tested in duplicates. The homogenates were independently prepared on three different days. Two of the homogenates were prepared with a Dounce homogenizer. The other two homogenates were prepared using a sonifier. $^{**}p < 0.05$, one-way ANOVA with Bonferroni's multiple comparison test

velocity enzyme for cUMP. Similar properties were previously observed for PDE7A that shows a 1000-fold lower substrate affinity, but a 6–7-fold higher V_{max} for cCMP as compared to cAMP (Monzel et al. 2014).

The K_M values determined for PDE3A-mediated cAMP hydrolysis (Table 1) are in good agreement with the K_M of 0.18 µM reported in the literature (Bender and Beavo 2006). The V_{max} values determined for cAMP degradation by recombinant PDE3A (Table 1) are close to the literature range of 3–6 µmol/min/mg (Bender and Beavo 2006), specifically when it is considered that, according to the manufacturer's specifications, our enzyme preparation contained up to 75% of non-PDE3A protein. This confirms that the recombinant enzyme used in our experiments is fully functional. It could be argued, however, that the observed cUMP hydrolysis was not caused by PDE3A, but by a contamination, because the manufacturer's specifications state a purity of only ≥25%, and our Western blots also show a major band between 70 and 100 kDa (Supplementary Fig. 1). However, the PDE3-selective inhibitor milrinone (Ito et al. 1988) potently inhibited cUMP hydrolysis in our samples, which strongly indicates the presence of PDE3A. Moreover, the K_i values for milrinone with respect to inhibition of PDE3A-mediated cUMP hydrolysis (Table 1) correspond well to the K_i value of 150 nM reported in the literature (inhibition of cardiac PDE3-mediated cAMP hydrolysis) (Ito et al. 1988). This suggests that cUMP

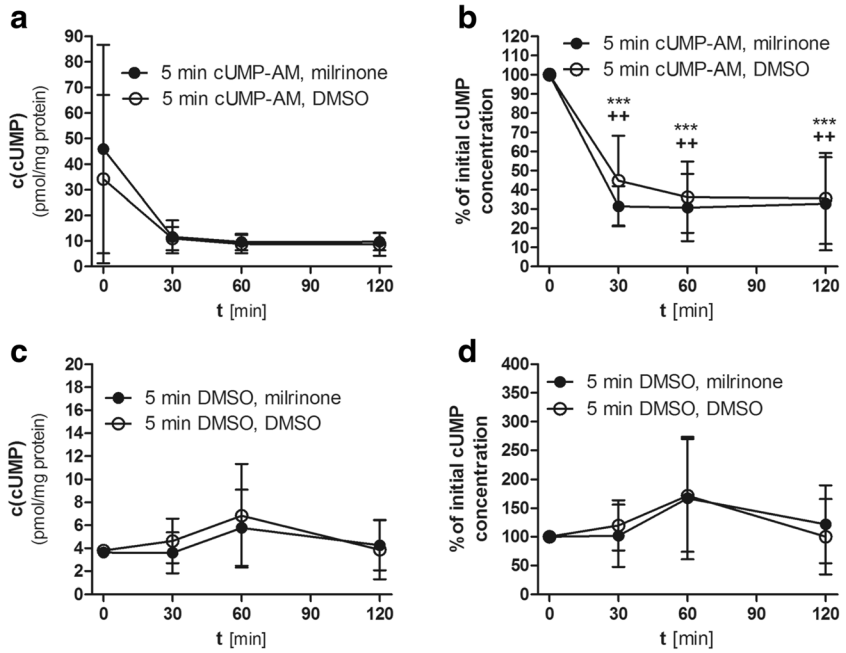

Fig. 7 Milrinone effect on cNMP metabolism in intact HL-1 cardiomyogenic cells. Cells were pre-incubated for 5 min with 10 µM of cUMP-AM (**a**, **b**) or with DMSO (negative controls; **c**, **d**). After removal of the cUMP-AM stimulus, intracellular concentrations of cUMP were determined by HPLC-MS/MS after 0, 30, 60, and 120 min in the presence (*filled symbols*) and in the absence (*open symbols*) of the PDE3 inhibitor milrinone. The figure shows absolute cUMP concentrations (**a**, **c**) as well as relative amounts normalized to the initial concentration at 0 min (**b**, **d**). Significance was calculated by one-way ANOVA followed by Dunnett's multiple comparison test (comparison of the 30, 60, and 120 min points to the 0 min (100%) point). *Asterisk* (*): statistics with milrinone-containing samples; *plus sign* (+): statistics with milrinone-free samples; *two symbols*: $p < 0.01$; *three symbols*: $p < 0.001$. Data are means ± SD from $n = 3$ (**c**, **d**) or $n = 4$ (**a**, **b**) independent experiments with single samples.

binds to the same site as cAMP and competes in a similar way with milrinone. It should be noted, however, that the milrinone K_i values calculated from inhibition of cAMP hydrolysis should be interpreted with caution. The high activity of PDE3A for cAMP caused complete hydrolysis of cAMP at low substrate concentrations, which may have led to an overestimation of K_i values.

PDE3A is a typical cardiac PDE (Meacci et al. 1992). Our qPCR data show that transcripts of the cUMP-degrading enzymes PDE3A, PDE3B, and PDE9A (Reinecke et al. 2011) are present in both HL-1 cells and nRCMs. It was, however, difficult to prove the presence of PDE3A on the protein level since the monoclonal rabbit anti-PDE3A antibody used in our experiments produced several non-specific signals at lower molecular masses (<100 kDa). The two bands observed around 130 kDa might belong to PDE3A isoforms. However, since a PDE3A-free cell line was not available as a negative control, it cannot be excluded that these bands represent non-specific staining of another protein that coincidentally has the "correct" molecular weight.

The presence of PDE3B and PDE9A mRNA is in agreement with previous reports on expression of PDE3B in mouse (Chung et al. 2015) and rat (Liu and Maurice 1998) cardiovascular tissues as well as of PDE9A in cardiac tissues (Kuhn 2015; Lee et al. 2015). In 1965, a cUMP-degrading PDE activity was detected in beef and dog heart tissue (Hardman and Sutherland 1965). Our results suggest that this activity may have been caused by PDE3A.This is supported by our data demonstrating cUMP-hydrolyzing activity in HL-1 cardiomyocyte homogenates that was completely eliminated by milrinone. Thus, cUMP hydrolysis by HL-1 cell homogenates is mainly caused by PDE3 rather than by the milrinone-insensitive PDE9A. Since milrinone does not differentiate between PDE 3A and 3B, however, we could not determine the individual contributions of the two PDE3 isoforms

In cUMP-loaded intact HL-1 cells, however, milrinone did not affect cUMP disposal. Independently of the presence of milrinone, intracellular cUMP quickly decreased within 30 min. It should be noted that cUMP pre-incubation occurred in the absence of inhibitor and milrinone was only added in the second phase. This was done to reach comparable intracellular cUMP starting concentrations for the degradation curves in the DMSO and milrinone samples. Milrinone, however, may require some time to inhibit PDE3A because it has to penetrate the cellular membrane first. Thus, despite the addition of milrinone, there could have been a time window during which PDE3A was able to hydrolyze cUMP. Alternatively, HL-1 cells may use a PDE-independent mechanism of cUMP removal. It is well established that the multidrug resistance proteins (MRPs) MRP4 and MRP5 are capable of exporting cNMPs like cAMP or cGMP (Ritter et al. 2005). In smooth muscle cells, export of cGMP by MRP4 is as important as hydrolysis by PDE5 and regulates cGMP-induced vascular smooth muscle relaxation (Krawutschke et al. 2015; Stangherlin and Zoccarato 2015). Non-canonical pyrimidine cNMPs are also substrates of MRP proteins. cCMP is exported by MRP5, while cUMP is transported by both MRP4 and 5 (Laue et al. 2014). MRP4 and 5 are also expressed in cardiac tissues (Dazert et al. 2003; Sassi et al. 2012). Thus, MRP-mediated transport may account for the observed PDE3-independent reduction of intracellular cUMP in HL-1 cells. This mechanism is supported by recently published data with HEL human erythroleukemia or K-562 human chronic myelogenous leukemia cells, where the unspecific MRP inhibitor probenecid strongly increased the apoptotic effects of several cNMPs including cUMP (Dittmar et al. 2016). Alternatively, the distinct effect of milrinone on HL-1 cell homogenates as compared to living HL-1 cells may be explained by PDE3 compartmentalization. Exogenously added cUMP-AM may deliver the cUMP to an intracellular compartment that does not contain PDE3.

Analysis of basal intracellular cNMP concentrations revealed that the cUMP concentration in HL-1 cells and nRCMs is comparable to that of the established second messenger cGMP. This is in good agreement with recent data describing the presence of cUMP in zebrafish heart (Dittmar et al. 2015). The low cUMP affinity of PDE3A and the lacking milrinone effect in intact HL-1 cells, however, suggest that PDE3A does not participate in homeostatic cUMP metabolism. Since cAMP has a several hundred-fold higher affinity to PDE3A than cUMP, the enzyme is most likely saturated by cAMP under physiological conditions, specifically because cardiomyocytes contain far more cAMP than cUMP. A low-affinity and high-velocity PDE for cUMP like PDE3A, however, may become important under pathophysiological conditions when intracellular cUMP concentration is dramatically increased. This is, for example, the case when the exotoxin ExoY is introduced into a host cell after infection with ExoY-positive *P. aeruginosa* (Beckert et al. 2014; Bähre et al. 2015). It should be noted that *P. aeruginosa* does not only infect the lung but is also involved in infective endocarditis that occurs in persons abusing intravenous drugs (Sousa et al. 2012).

In summary, we provide for the first time the basic enzyme kinetics parameters for PDE3A-mediated cUMP hydrolysis and show that HL-1 cardiomyogenic cells contain a milrinone-sensitive cUMP-degrading activity. Future studies should investigate interactions between the PDE3A substrates cAMP, cGMP, and cUMP in mixtures of these cNMPs that mimic intracellular conditions. Moreover, the experiment shown in Fig. 7 should be further developed and modified by changing the type and the concentration of the PDE inhibitor as well as the time when it is added to the sample. Moreover, the effect of MRP inhibitors like probenecid on extrusion of cUMP and other cNMPs by cardiomyocytes should be investigated. Furthermore, in addition to Hl-1 cells, cUMP metabolism of primary cardiomyocytes (e.g., neonatal or adult rat cardiomyocytes) should be analyzed. Finally, it would be interesting to examine if cUMP-degrading PDEs like PDE3A are upregulated in cells infected with ExoY-positive *P. aeruginosa* and if enhanced intracellular cUMP concentrations affect cardiomyocyte function.

Acknowledgements We thank Prof. Dr. Ralf Gerhard (Institute of Toxicology, MHH) and Dr. Sabine Wolter (Institute of Pharmacology, MHH) for excellent scientific discussions as well as Ms. Annette Garbe (Research Core Unit Metabolomics, MHH) and Ms. Martina Kasten (Molecular Cardiology Research Group, Department of Cardiology and Angiology, MHH) for outstanding technical support. Moreover, we thank the reviewers for their constructive comments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

Beckert U, Wolter S, Hartwig C, Bähre H, Kaever V, Ladant D, Frank DW, Seifert R (2014) ExoY from *Pseudomonas aeruginosa* is a nucleotidyl cyclase with preference for cGMP and cUMP formation. Biochem Biophys Res Commun 450:870–874. doi:10.1016/j.bbrc.2014.06.088

Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol Rev 58:488–520. doi:10.1124/pr.58.3.5

Bähre H, Danker KY, Stasch JP, Kaever V, Seifert R (2014) Nucleotidyl cyclase activity of soluble guanylyl cyclase in intact cells. Biochem Biophys Res Commun 443:1195–1199. doi:10.1016/j.bbrc.2013.12.108

Bähre H, Hartwig C, Munder A, Wolter S, Stelzer T, Schirmer B, Beckert U, Frank DW, Tümmler B, Kaever V, Seifert R (2015) cCMP and cUMP occur in vivo. Biochem Biophys Res Commun 460:909–914. doi:10.1016/j.bbrc.2015.03.115

Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Chung YW, Lagranha C, Chen Y, Sun J, Tong G, Hockman SC, Ahmad F, Esfahani SG, Bae DH, Polidovitch N, Wu J, Rhee DK, Lee BS, Gucek M, Daniels MP, Brantner CA, Backx PH, Murphy E, Manganiello VC (2015) Targeted disruption of PDE3B, but not PDE3A, protects murine heart from ischemia/reperfusion injury. Proc Natl Acad Sci U S A 112:E2253–E2262. doi:10.1073/pnas.1416230112

Claycomb WC, Lanson NA, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ (1998) HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci U S A 95:2979–2984

Dazert P, Meissner K, Vogelgesang S, Heydrich B, Eckel L, Böhm M, Warzok R, Kerb R, Brinkmann U, Schaeffeler E, Schwab M, Cascorbi I, Jedlitschky G, Kroemer HK (2003) Expression and localization of the multidrug resistance protein 5 (MRP5/ABCC5), a cellular export pump for cyclic nucleotides, in human heart. Am J Pathol 163:1567–1577. doi:10.1016/S0002-9440(10)63513-4

Dittmar F, Abdelilah-Seyfried S, Tschirner SK, Kaever V, Seifert R (2015) Temporal and organ-specific detection of cNMPs including cUMP in the zebrafish. Biochem Biophys Res Commun 468:708–712. doi:10.1016/j.bbrc.2015.11.020

Dittmar F, Wolter S, Seifert R (2016) Regulation of apoptosis by cyclic nucleotides in human erythroleukemia (HEL) cells and human myelogenous leukemia (K-562) cells. Biochem Pharmacol 112:13–23. doi:10.1016/j.bcp.2016.04.018

Göttle M, Dove S, Kees F, Schlossmann J, Geduhn J, König B, Shen Y, Tang WJ, Kaever V, Seifert R (2010) Cytidylyl and uridylyl cyclase activity of *Bacillus anthracis* edema factor and *Bordetella pertussis* CyaA. Biochemistry 49:5494–5503. doi:10.1021/bi100684g

Hardman JG, Sutherland EW (1965) A cyclic 3′,5′-nucleotide phosphodiesterase from heart with specificity for uridine 3′,5′-phosphate. J Biol Chem 240:3704–3705

Hartwig C, Bähre H, Wolter S, Beckert U, Kaever V, Seifert R (2014) cAMP, cGMP, cCMP and cUMP concentrations across the tree of life: high cCMP and cUMP levels in astrocytes. Neurosci Lett 579:183–187. doi:10.1016/j.neulet.2014.07.019

Hasan A, Danker KY, Wolter S, Bähre H, Kaever V, Seifert R (2014) Soluble adenylyl cyclase accounts for high basal cCMP and cUMP concentrations in HEK293 and B103 cells. Biochem Biophys Res Commun 448:236–240. doi:10.1016/j.bbrc.2014.04.099

Hilfiker-Kleiner D, Kaminski K, Kaminska A, Fuchs M, Klein G, Podewski E, Grote K, Kiian I, Wollert KC, Hilfiker A, Drexler H (2004) Regulation of proangiogenic factor CCN1 in cardiac muscle: impact of ischemia, pressure overload, and neurohumoral activation. Circulation 109:2227–2233. doi:10.1161/01.CIR.0000127952.90508.9D

Ito M, Tanaka T, Saitoh M, Masuoka H, Nakano T, Hidaka H (1988) Selective inhibition of cyclic AMP phosphodiesterase from various human tissues by milrinone, a potent cardiac bipyridine. Biochem Pharmacol 37:2041–2044

Krawutschke C, Koesling D, Russwurm M (2015) Cyclic GMP in vascular relaxation: export is of similar importance as degradation. Arterioscler Thromb Vasc Biol 35:2011–2019. doi:10.1161/ATVBAHA.115.306133

Kuhn M (2015) Cardiology: a big-hearted molecule. Nature 519:416–417. doi:10.1038/nature14373

Laue S, Winterhoff M, Kaever V, van den Heuvel JJ, Russel FG, Seifert R (2014) cCMP is a substrate for MRP5. Naunyn Schmiedeberg's Arch Pharmacol 387:893–895. doi:10.1007/s00210-014-1018-9

Lee DI, Zhu G, Sasaki T, Cho GS, Hamdani N, Holewinski R, Jo SH, Danner T, Zhang M, Rainer PP, Bedja D, Kirk JA, Ranek MJ, Dostmann WR, Kwon C, Margulies KB, Van Eyk JE, Paulus WJ, Takimoto E, Kass DA (2015) Phosphodiesterase 9A controls nitric-oxide-independent cGMP and hypertrophic heart disease. Nature 519:472–476. doi:10.1038/nature14332

Liu H, Maurice DH (1998) Expression of cyclic GMP-inhibited phosphodiesterases 3A and 3B (PDE3A and PDE3B) in rat tissues: differential subcellular localization and regulated expression by cyclic AMP. Br J Pharmacol 125:1501–1510. doi:10.1038/sj.bjp.0702227

Meacci E, Taira M, Moos M, Smith CJ, Movsesian MA, Degerman E, Belfrage P, Manganiello V (1992) Molecular cloning and expression of human myocardial cGMP-inhibited cAMP phosphodiesterase. Proc Natl Acad Sci U S A 89:3721–3725

Monzel M, Kuhn M, Bähre H, Seifert R, Schneider EH (2014) PDE7A1 hydrolyzes cCMP. FEBS Lett 588:3469–3474. doi:10.1016/j.febslet.2014.08.005

Niiya T, Osawa H, Onuma H, Suzuki Y, Taira M, Yamada K, Makino H (2001) Activation of mouse phosphodiesterase 3B gene promoter by adipocyte differentiation in 3T3-L1 cells. FEBS Lett 505:136–140

Omori K, Kotera J (2007) Overview of PDEs and their regulation. Circ Res 100:309–327. doi:10.1161/01.RES.0000256354.95791.f1

Reinecke D, Burhenne H, Sandner P, Kaever V, Seifert R (2011) Human cyclic nucleotide phosphodiesterases possess a much broader substrate-specificity than previously appreciated. FEBS Lett 585:3259–3262. doi:10.1016/j.febslet.2011.09.004

Ritter CA, Jedlitschky G, Meyerzu Schwabedissen H, Grube M, Köck K, Kroemer HK (2005) Cellular export of drugs and signaling molecules by the ATP-binding cassette transporters MRP4 (ABCC4) and MRP5 (ABCC5). Drug Metab Rev 37:253–278. doi:10.1081/DMR-200047984

Sassi Y, Abi-Gerges A, Fauconnier J, Mougenot N, Reiken S, Haghighi K, Kranias EG, Marks AR, Lacampagne A, Engelhardt S, Hatem SN, Lompre AM, Hulot JS (2012) Regulation of cAMP homeostasis

by the efflux protein MRP4 in cardiac myocytes. FASEB J 26:1009–1017. doi:10.1096/fj.11-194027

Seifert R (2015) cCMP and cUMP: emerging second messengers. Trends Biochem Sci 40:8–15. doi:10.1016/j.tibs.2014.10.008

Seifert R, Schneider EH, Bähre H (2015) From canonical to non-canonical cyclic nucleotides as second messengers: pharmacological implications. Pharmacol Ther 148:154–184. doi:10.1016/j.pharmthera.2014.12.002

Sousa C, Botelho C, Rodrigues D, Azeredo J, Oliveira R (2012) Infective endocarditis in intravenous drug abusers: an update. Eur J Clin Microbiol Infect Dis 31:2905–2910. doi:10.1007/s10096-012-1675-x

Stangherlin A, Zoccarato A (2015) Relax: it's not all about degradation. Arterioscler Thromb Vasc Biol 35:1907–1909. doi:10.1161/ATVBAHA.115.306217

Taira M, Hockman SC, Calvo JC, Belfrage P, Manganiello VC (1993) Molecular cloning of the rat adipocyte hormone-sensitive cyclic GMP-inhibited cyclic nucleotide phosphodiesterase. J Biol Chem 268:18573–18579

Wechsler J, Choi YH, Krall J, Ahmad F, Manganiello VC, Movsesian MA (2002) Isoforms of cyclic nucleotide phosphodiesterase PDE3A in cardiac myocytes. J Biol Chem 277:38072–38078. doi:10.1074/jbc.M203647200

Wolter S, Golombek M, Seifert R (2011) Differential activation of cAMP- and cGMP-dependent protein kinases by cyclic purine and pyrimidine nucleotides. Biochem Biophys Res Commun 415:563–566. doi:10.1016/j.bbrc.2011.10.093

Wolter S, Kloth C, Golombek M, Dittmar F, Försterling L, Seifert R (2015) cCMP causes caspase-dependent apoptosis in mouse lymphoma cell lines. Biochem Pharmacol 98:119–131. doi:10.1016/j.bcp.2015.08.096

Zong X, Krause S, Chen CC, Krüger J, Gruner C, Cao-Ehlker X, Fenske S, Wahl-Schott C, Biel M (2012) Regulation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channel activity by cCMP. J Biol Chem 287:26506–26512. doi:10.1074/jbc.M112.357129