Optogenetic manipulation of cyclic guanosine monophosphate to probe phosphodiesterase activities in megakaryocytes

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Cyclic guanosine monophosphate (cGMP) signalling plays a fundamental role in many cell types, including platelets. cGMP has been implicated in platelet formation, but mechanistic detail about its spatio-temporal regulation in megakaryocytes (MKs) is lacking. Optogenetics is a technique which allows spatio-temporal manipulation of molecular events in living cells or organisms. We took advantage of this method and expressed a photo-activated guanylyl cyclase, Blastocladiella emersonii Cyclase opsin (BeCyclop), after viral-mediated gene transfer in bone marrow (BM)-derived MKs to precisely light-modulate cGMP levels. BeCyclop-MKs showed a significantly increased cGMP concentration after illumination, which was strongly dependent on phosphodiesterase (PDE) 5 activity. This finding was corroborated by real-time imaging of cGMP signals which revealed that pharmacological PDE5 inhibition also potentiated nitric oxide-triggered cGMP generation in BM MKs. In summary, we established for the first-time optogenetics in primary MKs and show that PDE5 is the predominant PDE regulating cGMP levels in MKs. These findings also demonstrate that optogenetics allows for the precise manipulation of MK biology.

Highlights
— Establishment of optogenetics in primary MKs
— Illumination of YFP-BeCyclop megakaryocytes results in a significant cGMP increase
— Phosphodiesterase 5 hydrolyses elevated cGMP in megakaryocytes in several minutes

1. Introduction

Binding of nitric oxide (NO) to guanylyl cyclases increases the cyclic guanosine monophosphate (cGMP) formation. cGMP is a key intracellular signalling molecule in many cell types and tissues and exerts multiple cellular effects via its downstream effectors, such as protein kinase G (PKG) or cyclic nucleotide-gated channels [1]. The cGMP signalling pathway is long known for its critical role in the maintenance of cardiovascular homeostasis. Fluorescent cGMP biosensors have emerged as powerful tools for the sensitive analysis of cGMP pathways at the single-cell level [2], and methods that allow for precise

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manipulation of cGMP levels by light are being developed [3]. The NO/cGMP/PKG pathway is highly expressed in platelets and its activation has been linked to platelet inhibition [4]. Analysis of spatio-temporal dynamics of platelet cGMP using cGMP sensor mice revealed that cGMP generation is increased in shear-exposed platelets at the periphery of a thrombus thereby limiting thrombus growth [5]. Cellular cGMP levels are regulated by phosphodiesterases (PDEs), which catalyse the hydrolysis of the 3' phosphate bond of cGMP to generate 5' GMP. Platelets express PDE2, 3 and 5, of which each is able to degrade cGMP [4–7]. Bone marrow (BM) megakaryocytes (MKs) are the immediate progenitor cells of blood platelets [8]. Studies on MKs derived from mouse fetal liver cells (FLCs) showed that PDE3A, PDE4A1 and PDE5 are detectable in maturing MKs, while PDE2A is found in the non-MK fraction, and that in vitro platelet release is enhanced by cGMP [9]. However, the spatio-temporal regulation of cGMP signals by PDEs in MKs has not been addressed due to the lack of appropriate tools. Therefore, we expressed a light-sensitive guanylyl cyclase in primary BM-derived MKs to tightly manipulate cGMP levels [3]. We demonstrate that the cGMP concentration in these MKs can be increased upon illumination, which rapidly declines in the dark caused by PDE5 activity.

2. Results and discussion

Concentration-dependent increase of cGMP in BM-derived MKs was measured when cells had been incubated with riociguat, a pharmacological stimulator of the NO-sensitive guanylyl cyclase (figure 1a). To tightly manipulate cGMP levels, the photo-activated guanylyl cyclase opsin from Blastocladiella emersonii, BeCyclop, was expressed in BM-derived MKs after viral transduction, which allows light-triggered cGMP increase as shown in heterologous cells and Caenorhabditis elegans [3]. Localization of YFP-BeCyclop in the membrane system of BM-derived MKs was confirmed by confocal microscopy (figure 1c). Illumination (green light: 520 nm) of YFP-BeCyclop-expressing MKs were illuminated with green light (520 nm) for 5 min and immediately lysed or after 3 min in the dark. (f) The expression of PDE3 and PDE5 in BM-derived MKs and platelets. GAPDH served as control.

Figure 1. Optogenetic manipulation of cGMP levels in BM-derived MKs. (a) BM-derived MKs were incubated for 10 min with 1, 5, 10 or 50 µM riociguat. DMSO was used as vehicle control. (n = 3, representative for two independent experiments). (b) A schematic model of the light-gated guanylyl cyclase, BeCyclop. (c) Representative images (microscope: Leica SP8) of YFP-BeCyclop expression in BM-derived MKs. MK/platelet-specific membrane protein GPIX. (d) BM-derived YFP-BeCyclop-expressing MKs were illuminated with green light (520 nm) for 5 min and immediately lysed. Untransduced cells or no illumination served as controls. (n = 3, representative for two independent experiments). (e) YFP-BeCyclop-expressing MKs were illuminated with green light (520 nm) for 5 min and immediately lysed or after 3 min in the dark. (f) The expression of PDE3 and PDE5 in BM-derived MKs and platelets. GAPDH served as control.
Be zaprinast (Zapr.): 40 µM, IBMX: 100 µM, milrinone (Milr.): 20 µM, BAY 60-7550: 10 µM, sildenafil: 100 µM. (ing cGMP concentration. DMSO control in (b), H2O control in (c). (n = 3). Vinpocetine (Vinp.): 20 µM, EHNA: 10 µM, vardenafil (Vard.): 50 µM, TAK-063: 0.15 µM, zaprinast (Zapr.): 40 µM, IBMX: 100 µM, milrinone (Milr.): 20 µM, BAY 60-7550: 10 µM, sildenafil: 100 µM. (d) The determination of cAMP concentration of YFP-BeCyclop-expressing BM-derived MKs after incubation with forskolin or illumination with green light for 5 min (n = 3).

Most relevant PDEs and found a strong expression of PDE5 and only a weak expression of PDE3 in BM-derived MKs and platelets (figure 1f). This data is in agreement with the copy number analysis of the murine platelet proteome, which revealed a high PDE5 expression in murine platelets [10]. Therefore, cells were pretreated with the specific PDE5 inhibitor tadalafil, which resulted in a significant but only subtle cGMP increase (DMSO: 1.02 ± 0.15 pmol ml⁻¹; tadalafil: 1.34 ± 0.09 pmol ml⁻¹; figure 2a). However, cGMP levels were 6.9-fold increased when pretreated with tadalafil and 5 min illuminated (20.45 ± 4.3 pmol ml⁻¹), as compared to illumination without tadalafil pretreatment (2.95 ± 0.45 pmol ml⁻¹). These data strongly suggest that PDE5 limits the cGMP concentration increase in MKs. Furthermore, the inhibition of PDE5 resulted in less cGMP decrease to 10 ± 1.5 pmol ml⁻¹ in 3 min after illumination (figure 2a), again confirming that PDE5 degrades cGMP in MKs. In agreement with our findings with tadalafil, illuminated MKs preincubated with other PDE5 inhibitors (vardenafil: PDE5, 6; sildenafil: PDE5, 6) also significantly increased cGMP concentration compared to illuminated YFP-BeCyclop-MKs without inhibitor (figure 2b,c), whereas non-PDE5 inhibitors did not elevate intracellular cGMP (vinpocetine: PDE1; EHNA: PDE2; BAY60-7550: PDE2; milrinone: PDE3, 4; TAK-063: PDE10; figure 2b). Interestingly, also the broad-spectrum PDE inhibitors zaprinast (PDE5, 6, 9, 10, 11) and IBMX (PDE1, 2, 3, 4, 5, 7, 11) had no or only minor effects, respectively (figure 2b).

To demonstrate the high specificity of BeCyclop, MKs expressing YFP-BeCyclop were either stimulated with the adenylyl cyclase activator forskolin or illuminated and subsequently, cyclic adenosine monophosphate (cAMP) was determined. While forskolin significantly increased the cAMP concentration in MKs, illumination had no effect on cAMP concentration, demonstrating that BeCyclop cannot produce or influence cAMP (figure 2d).

Next, we used MK/platelet-specific cGMP sensor mice to spatio-temporally visualize cGMP dynamics in MKs ex vivo (figure 3a) [5,11]. Isolated femurs with externalized BM were superfused with 1 ml min⁻¹ imaging buffer. In contrast with static cell culture experiments, this method allows to rapidly add and remove the pharmacological compounds within seconds similar to optogenetic experiments. Application of the NO donor diethylamine NONOate (DEA/NO) increased cGMP in BM MKs (figure 3b,c). Similar to the optogenetic results, the PDE5-specific inhibitor tadalafil alone slowly increased cGMP and significantly augmented the NO-induced cGMP signal in MKs (figure 3b,c). These data strongly suggest that BM MKs in their native environment express a functional NO/cGMP/PDE5 signalling pathway that can be pharmacologically enhanced with PDE5 inhibitors.
Optogenetics has become successful as an easily applicable tool for precise spatio-temporal manipulation of molecular events in living cells or organisms. By now, optogenetic applications have expanded from neuroscience to other fields such as the cardiovascular system. We established to the best of our knowledge the first-time optogenetics in primary MKs. By expressing the photo-activated guanylyl cyclase, BeCyclop, we tightly controlled cGMP levels in MKs by light. We show that PDE5 is the major PDE, counteracting cGMP levels in BM-derived MKs, and could verify our optogenetic results by cGMP imaging in BM MKs ex vivo using cGMP sensor mice. Our reversible approaches allowing to trigger the intracellular process and directly analyse the effect in a high spatio-temporal resolution revealed that PDE5 is highly active in BM-derived MKs as cGMP levels rapidly declined after removing the stimulus. Our findings are corroborated by research on mouse FLK-derived MKs showing that PDE5 expression is highest in mature FLK-derived MKs [9]. Our study shows that optogenetics in MKs allows to light-modulate the function of key signalling molecules thereby possibly identifying novel regulatory mechanisms of MK maturation and platelet production. However, our study has also limitations as the transduction efficiency of MKs was only about 30%. We analysed the complete MK fraction since complicated sorting of mature MKs (cell diameter of approx. 35 µm) probably leads to light-activation before starting the experiment. Thus, our data even underestimate the light-induced increase of cGMP in single MKs expressing YFP-BeCyclop due to the untransduced cell fraction. Further studies are required using MK/platelet-specific optogenetic mouse lines to spatio-temporally light-manipulate molecules or proteins in MKs and platelets in vivo. The finding that BM MK express PDE5 also informs future therapeutic strategies to increase cGMP concentrations and potentially modulate platelet biogenesis with clinically used PDE5 inhibitors.

3. Experimental procedures

3.1. Animals

MK/platelet-specific cGMP sensor mice (cGi500-L2Cre<sup>fl/fl</sup>; Pf4-Cre<sup>fl/fl</sup> [12]) were used to visualize cGMP signals in MKs in real time [5,11]. Mouse BM isolation was conducted according to the guidelines of the local governments.

3.2. Reagents

Riociguat (Selleck Chemicals), tadalafil (Sigma-Aldrich/Supelco), DMSO, forskolin (Sigma-Aldrich), vinpocetine, EHNA (Enzo Life Sciences), BAY60-7550, TAK-063 (Cayman Chemical), milrinone, vardenafil, zaprinast (Santa Cruz Biotechnology), sildenafil (Merck), IBMX (Thermo Fisher Scientific) and DEA/NO (Axxora).

3.3. Bone marrow-derived megakaryocytes

BM cells were obtained from femur and tibia of C57BL/6 mice by flushing, and lineage depletion was performed using an antibody cocktail of anti-mouse CD3, clone 17A2; anti-mouse Ly-6G/Ly-6C, clone RB6-8C5; anti-mouse CD11b, clone M1/70; anti-mouse CD45R/B220, clone RA3-6B2; anti-mouse TER-119/Erythroid cells, clone Ter-119 [1.5 µg of each antibody per mouse, Biocool (Germany)] and magnetic beads (Dynabeads Untouched Mouse CD4 Cells, Invitrogen). Lineage-negative (Lin<sup>-</sup>) cells were cultured in DMEM medium (supplemented with 4 mM L-glutamine, 100 U ml<sup>-1</sup> penicillin, 50 mg ml<sup>-1</sup> streptomycin containing 1% of recombinant TPO (homemade) and 100 U ml<sup>-1</sup> recombinant Hirudin (Hyphen Biomed) at 37°C under 5% CO<sub>2</sub>.

3.4. Expression of YFP-BeCyclop in megakaryocytes

YFP-BeCyclop DNA was cloned into the murine stem cell virus vector and transfected into 293T cells. Viral supernatant
was collected and BM-derived cells were infected on day 1 [13]. A bovine serum albumin density gradient was used at culture day 3 to separate MKs from non-MK cells. Experiments were performed on day 4.

3.5. Illumination and determination of cyclic guanosine monophosphate concentration

Cell density was adjusted to $5 \times 10^4$ cells ml$^{-1}$. If needed, samples were preincubated with agonist or inhibitor for 10 min at room temperature. Subsequently, samples were kept in the dark or illuminated with light (520 nm, 20 µW mm$^{-2}$) for 5 min at room temperature and lysed either immediately or after 3 min in the dark with 0.1 M hydrogen chloride. Concentrations of cGMP or cAMP were measured by DetectX Direct cyclic GMP or AMP Enzyme Immunoassay Kit, respectively, and analysed by the optical density at 450 nm with a Thermo Scientific Multiskan microplate spectrophotometer.

3.6. Immunoblotting

MK and platelet lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. Membranes were incubated with an anti-PDE3A antibody (GeneTex) or an anti-PDE5 antibody (Abcam) overnight. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence solution (MoBiTec) were used for visualization. Immunoblots were recorded directly using an Amersham Imager 600 (GE Healthcare).

3.7. Real-time cyclic guanosine monophosphate imaging in megakaryocytes ex vivo

Femurs and tibiae were dissected from MK/platelet-specific cGMP sensor mice (cGi500-L2flo; Pf4-Cre tg/+ [12]). Bone tissue was carefully removed with scissors and forceps on one side to give access to the BM. Opened bones were placed in a superfusion chamber (RC 26, Warner Instruments), mounted with a Slice Hold-Down (SHD 26H/10, Warner Instruments) and continuously superfused with imaging buffer (140 mM NaCl, 5 mM KCl, 1.2 mM MgCl$_2$, 2 mM CaCl$_2$, 5 mM HEPES and 10 mM D-glucose; pH = 7.4) with or without added drugs. Real-time FRET/cGMP imaging was performed by recording CFP and YFP fluorescence as described in detail elsewhere [14]. The relative CFP/YFP ratio change (black trace in the respective graph; referred to as R (cGMP)) correlates with the cGMP concentration change.

3.8. Data analysis

Results are shown as mean ± s.e.m. Statistical significance was assessed by an unpaired $t$-test with GraphPad Prism software. $p$-values < 0.05 were considered significant (***<0.0001; **<0.001; *<0.01 to 0.05; *0.01 to 0.05). ns: non-significant. Statistical analysis of cGMP sensor imaging data was performed with Origin 2019 (OriginLab, Northampton, MA, USA). Since datasets were not normally distributed, statistical differences were analysed non-parametrically by Mann-Whitney U-test.

Ethics. Mouse bone marrow isolation was conducted according to the guidelines of the local governments.

Data accessibility. Any additional information required to reanalyse the data reported in this paper is available from the corresponding author upon request.

Authors’ contributions. Y.Z.: data curation, investigation, methodology and writing—review and editing; P.B.: data curation, investigation, methodology and writing—review and editing; D.S.: data curation, investigation, methodology and writing—review and editing; S.G.: data curation and methodology; H.K.: investigation and methodology; S.F.: funding acquisition, investigation, methodology, supervision and writing—review and editing; G.N.: funding acquisition, methodology, project administration, supervision and writing—review and editing; R.F.: funding acquisition, project administration, supervision and writing—review and editing; S.G.: funding acquisition, project administration, supervision and writing—original draft; M.B.: conceptualization, funding acquisition, project administration, supervision and writing—original draft.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This work was supported by TR240 grant with project no. 374031971 of the Deutsche Forschungsgemeinschaft (DFG; German Research Foundation).

Acknowledgements. The authors thank Sarah Seidel and Dr Markus Spindler for the help with experiments.

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