How to control cyclic nucleotide signaling by light

Vera Jansen*[1] , Jan F. Jikeli*[1], Dagmar Wachten[1,2]

[1]Center of Advanced European Studies and Research (caesar), Minerva Max Planck Research Group, Molecular Physiology, Bonn, Germany

[2]Institute of Innate Immunity, University Hospital, University of Bonn, Bonn, Germany

To whom correspondence should be addressed:

PD Dr. Dagmar Wachten, Center of Advanced European Studies and Research (caesar), Ludwig-Erhard-Allee 2, 53175 Bonn, Germany; phone: +49-228-9656-311, fax: +49-228-9656-9311, email: dagmar.wachten@caesar.de

* equally contributed

Abstract

Optogenetics allows to non-invasively manipulate cellular functions with spatio-temporal precision by combining genetic engineering with the control of protein function by light. Since the discovery of channelrhodopsin has pioneered the field, the optogenetic toolkit has been ever expanding and allows now not only to control neuronal activity by light, but rather a multitude of other cellular functions. One important application that has been opened up in recent years is the light-dependent control of second messenger signaling. The optogenetic toolkit now allows to control cyclic nucleotide-dependent signaling by light *in vitro* and *in vivo*.

Introduction

Optogenetics describes a technique to genetically engineer a cell with light-sensitive proteins and, thereby, control cellular function with spatio-temporal precision. The discovery of channelrhodopsin 1 and 2 (ChR1, ChR2) as light-gated ion channels [1,2] and their application to control the activity of excitable cells by light paved the way for this new research field [3-8].

The core of every optogenetic tool is the light-sensitive domain that changes protein conformation upon photon absorption (Figure 1). In case of the ChRs, this photosensory domain is rhodopsin-based, consisting of an opsin apoprotein covalently linked to the chromophore retinal, which absorbs blue to green light (450-545 nm) [9]. So far, two major rhodopsin classes have been identified: Type I microbial rhodopsins, which can be e.g. ion channels like ChR, and type II animal rhodopsins, which are G-protein-coupled-receptors e.g. in the visual system [9]. The flavin-containing photoreceptors of the LOV (light, oxygen, voltage sensing), the BLUF (blue light-utilizing flavin adenine dinucleotide), and the cryptochrome family further extend the repertoire of photosensory domains [10,11]. All three members utilize a flavin-derived cofactor, absorbing light in the UV to green/blue light range (320-500 nm): LOV domains mostly contain a flavin mononucleotide (FMN), whereas BLUF domains and cryptochromes contain a flavin adenine dinucleotide (FAD) with both, FAD and FMN, being available in all eukaryotic cells. Most importantly from an optogenetic point of view, BLUF and LOV domains are natively connected to a number of effector domains, which convey e.g. enzymatic activity or DNA- and protein-binding [10]. However, the activation of BLUF- or LOV-domains by blue light complicates optogenetic *in vivo* applications due to its low tissue penetration and higher phototoxicity compared to e.g. red light [12]. These shortcomings can be overcome by using phytochromes, which sense light in the red/far-red range (600-800 nm) and covalently bind a tetrapyrrole (bilin) chromophore. Phytochromes from bacteria (also called bacteriophytochromes) bind biliverdin Ixα, which is present in eukaryotic cells [13], whereas plant phytochromes bind phycocyanobilin or phytochromobilin, which have to be added exogenously in e.g. mammalian cells or produced via heterologous expression of the synthesis machinery [14,15].

Different effector domains are coupled to the photosensory domains described above, with one important class being enzymes that produce the cyclic nucleotides cAMP (3´,5´-cyclic adenosine monophosphate) and cGMP (3´,5´-cyclic guanosine monophosphate) (Figure 1). Cyclic nucleotides are ubiquitous second messengers across phyla, controlling a variety of physiological functions. Cyclic nucleotide signaling is highly organized in the cell, creating microdomains that fulfil different functions and that are differentially regulated [16]. Due to the lack of suitable tools, it has been rather difficult to analyze these microdomains in great detail. However, optogenetic manipulation of cyclic nucleotide signaling has now opened

up new avenues to fulfil this need. Here, we give an overview on recent developments concerning the optogenetic toolkit to control cAMP and cGMP dynamics *in vitro* and *in vivo*.

Light-activated adenylate cyclases

The first photoactivated adenylate cyclase (AC) to be identified has been from the unicellular flagellate *Euglena gracilis* [17]. The enzyme was termed euPAC and was the first optogenetic tool that has been applied to directly increase cAMP [18] (Figure 1). The enzyme is a tetramer, consisting of two subunits, PACα and PACβ, which both contain two BLUF domains and two catalytic domains. Both subunits can be expressed individually and display light-activated cyclase activity. However, PACα shows a high basal (dark) activity, but its light-stimulated activity is 100-fold higher than for PACβ [18]. Light-dependent activity of both proteins has been demonstrated in *Xenopus laevis* oocytes and in HEK293 cells. PACα has also been used *in vivo* in transgenic flies (*Drosophila melanogaster*), expressing the protein exclusively in the brain [18] and in the marine gastropod *Aplysia*, expressing euPAC in specific neurons that control learning behavior [19]. Although both approaches allowed to control animal behavior, the applications were rather limited. Ubiquitous expression of PACα in flies caused a lethal cAMP increase due to its high dark activity [18] and in combination with the rather large size of the protein, it was difficult to apply euPAC in other model systems. Both limitations could be overcome by using *BlaC*, which was identified by the Gomelsky group in the genome of the bacterium *Beggiatoa* [20] (Figure 1). BlaC is less than half the size of euPAC because it contains only one BLUF and one type III AC catalytic domain. Of note, all bacterial type III ACs are functional as dimers [21]. In parallel, the Hegemann group published their results for the same protein, which they called bPAC [22]. They demonstrated a 300-fold increase in cyclase activity after blue light-stimulation, which decays thermally within 20 s [22]. They not only characterized the purified protein, but also applied bPAC in *Xenopus* oocytes, in rat hippocampal pyramidal cells, and *in vivo* by generating transgenic flies. bPAC outperformed euPAC in three respects: 1. bPAC is smaller and a dimer, 2. bPAC displays a larger dynamic range and a reduced dark activity, 3. bPAC contains only one BLUF domain, making modifications of photoreceptor kinetics more straightforward. In fact, key residues for light regulation of the enzyme have already been identified and mutated [23,24], with one of them being a mutant with a slightly red-shifted absorbance and a decreased dark activity [24]. Thus, bPAC has become the tool of choice for the following applications using PACs to increase intracellular cAMP levels. Application of bPAC in different cell types of the *Drosophila* renal tube revealed separate roles of PKA and Epac in renal function [25]. Introducing bPAC into the intracellular parasite *Toxoplasma gondii* demonstrated that the parasite-derived cAMP production is important for stage differentiation and host-cell invasion [26]. In zebrafish, bPAC has been used to control glucocorticoid levels, e.g. cortisol, while studying the behavior of the freely-moving animal [27-29]. The first transgenic mouse line expressing bPAC has been

generated by our laboratory, in which we restricted bPAC expression to sperm flagella [30] (Figure 1). Mammalian sperm crucially rely on cAMP synthesis for motility. Sperm lacking their principal cAMP source, the soluble adenylate cyclase SACY, are immotile and cannot fertilize the egg [31-33]. Blue light stimulation of transgenic sperm increased intracellular cAMP levels and, in turn, elevated the flagellar beat frequency. Furthermore, light-dependent activation of bPAC in immotile sperm lacking SACY restored flagellar motility and, thereby, enabled sperm to fertilize oocytes *in vitro* [30], demonstrating that such a fundamental process as fertilization can be controlled by optogenetics.

A PAC that relies on a LOV domain as a photoreceptor domain has been identified in the cyanobacterium *Microcoleus chthonoplastes* and has therefore been named mPAC [34] (Figure 1). mPAC exhibited relatively high dark activity (compared to bPAC) and its activity was amplified 30-fold after blue light-stimulation [35]. Expression of mPAC in the amoeba *Dictyostelium discoideum* restored, at least partially, development after light stimulation in mutants lacking the endogenous AC [35].

In addition to naturally occurring molecules, there has been an effort to selectively engineer optogenetic tools for manipulating cyclic nucleotides: by fusing a photosensory module of the *Rhodobacter sphaeroides* bacteriophytochrome and the AC catalytic domain from the bacterium *Nostoc* sp. CyaB1, a PAC termed IlaC has been generated [36] (Figure 1). When introducing IlaC into cholinergic neurons of *Caenorhabditis elegans*, irradiation with 700 nm light increased intracellular cAMP levels and allowed to control worm behavior [36]. However, animals were already more active when cultivated under ambient light conditions. To allow efficient light-stimulated activity, animals had to be maintained in the dark for a whole generation. Thus, while red-light stimulation has the advantage of deeper tissue penetration, it suffers from increased pre-stimulation under ambient light conditions. The latter could be overcome by illumination using far-red light.

Light-activated guanylate cyclases

The first approach to design a photoactivated guanylate cyclase (GC) was based on the *Beggiatoa* BlaC/bPAC protein by selectively mutating amino acids that are specific for ACs into amino acids that are specific for GCs [20]. A triple mutant (K197E/D265K/T267G) displayed a 10-fold higher GC than AC activity and was designated BlaG [20] (Figure 1). BlaG was also applied *in vivo* and termed EROS (erectile optogenetic stimulator) [37]. EROS expression in rats was used to induce a light-activated penis erection by cGMP-dependent relaxation of the *corpus cavernosum* smooth muscle and subsequent blood influx [37] (Figure 1). However, it is questionable whether a light-dependent approach is a major advancement for the therapy of erectile dysfunction compared to the existing therapy based on Viagra, an

oral cGMP-specific phosphodiesterase inhibitor [37]. A natural photoactivated GC, BeGC1, was later isolated from the fungus *Blastocladiella emersonii.* It consists of a GC catalytic domain and a rhodopsin photosensory domain [38]. The protein was applied as an optogenetic tool in parallel in the Hegemann and Nagel/Gottschalk labs, where they called the protein either RhGC [39] or BeCyclOP [40], respectively (Figure 1). RhGC expression in *Xenopus* oocytes, Chinese hamster ovary cells, or primary neurons increased cGMP levels after green light-stimulation on the subsecond timescale [39]. BeCyclOP expression in O_2/CO_2 sensory neurons of *C. elegans* was used to evoke a behavioral response independent of a sensory stimulus [40]. Showing only very low dark activity and a high dynamic range (>1000-fold increase in activity upon light stimulation), RhGC/BeCyclOP is perfectly suited to control intracellular cGMP levels and physiological functions by light.

Light-activated phosphodiesterases

To gain even closer control over intracellular cAMP/cGMP levels, not only photoactivated cyclases, but also photoactivated phoshodiesterases for degradation of cAMP/cGMP are needed. To date, no naturally occurring photoactivated cyclic nucleotide-specific phosphodiesterase has been identified. However, the Möglich group succeeded in engineering a light-activated phosphodiesterase (LAPD) by combining the photosensory domain of a bacterial phytochrome from *Deinococcus radiodurans* and the catalytic domain of the human phosphodiesterase 2A (PDE2A) [41] (Figure 1). Stimulation with red light up-regulated cAMP/cGMP hydrolysis up to 6-fold, whereas far-red light was used to down-regulate LAPD activity. Although the kinetics for the reverse transition are slow, the inactivation is not merely due to thermal reversion of the chromophore [41]. Light-dependent activation of LAPD has been demonstrated in mammalian cell culture and in zebrafish embryos [41]. Future studies will reveal whether 1. biliverdin is readily available in all model organisms and cell types, allowing a broad application of a bacterial phytochrome-based optogenetic tool like LAPD, 2. the specificity for cAMP or cGMP can be optimized, and 3. the dynamice range, reversibility, and efficiency can be improved.

Conclusion

Optogenetics is a field that has revolutionized not only basic research, but also makes its way into medical sciences and clinical applications. In basic research, the spatio-temporal control of signaling pathways allows to interrogate the function of subcellular domains and organelles. Approaches to target optogenetic tools to subcellular compartments are currently being implemented, unraveling cellular dynamics on a whole new level. An orthogonal design of new optogenetical tools by combining different photosensory and effector domains will open up new avenues to control cellular signaling and function by light. In medical sciences, current strategies heavily rely on ChRs. Although the cyclic nucleotide-based optogenetic toolkit has

proven its use in animal models, clinical applications for optogenetics in general are lacking so far. A powerful approach for future studies will be the combination of optogenetics with state-of-the-art genome editing tools like the CRISPR-Cas9-System. The outcome of this endeavor is difficult to predict, but we are looking forward to an exciting scientific future with optogenetics being a prominent player.

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Conflict of interest

The authors declare no competing financial interests.

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    The anterior pituitary is the major link between nervous and hormonal systems, which allow the brain to generate adequate and flexible behavior. Targeting bPAC to pituitary corticotroph cells in zebrafish allowed to selectively enhance corticotroph cell activity and reveal that pituitary corticotroph cells can rapidly modulate avoidance behaviors on the onset of stress.

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    They manipulate endogenous concentrations of cortisol in the zebrafish by targeting bPAC to interrenal cells using a promoter element of the steroidogenic acute regulatory protein and, thereby, studying the effects of hypercortisolemia.

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    This study is the first to generated transgenic mice expressing bPAC and show that optogenetics can be used to control sperm function and, thereby, fertilization by light. Transgenic mice expressing bPAC exclusively in sperm were crossed with mice lacking the endogenous enzyme for cAMP synthesis in sperm. Sperm of these mice are usually non-motile, and the mice consequently infertile. However, after stimulation with blue light, sperm produced cAMP, started to swim again, and were even able to fertilize eggs.

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They engineered adenylate cyclases to be regulated by light in the near-infrared spectral window using the photosensory module of the *Rhodobacter sphaeroides* bacteriophytochrome BphG1 and the adenylate cyclase domain from *Nostoc sp.* CyaB1. They produced an enzyme with a more stable photoactivated state and a sixfold photodynamic range. When expressed in cholinergic neurons in *Caenorhabditis elegans*, the engineered adenylate cyclase affected worm behavior in a light-dependent manner.

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They generated an engineered a blue light-stimulated guanylate cyclase producing cGMP in mammalian cells. They expressed this tool in the *corpus cavernosum* of rats and called it EROS (erectile optogenetic stimulator). Blue light stimulation of EROS-transfected *corpus cavernosum* enabled penile erection associated with occasional ejaculation. Photostimulated short-circuiting of complex psychological, neural, vascular, and endocrine factors to stimulate penile erection in the absence of sexual arousal may foster novel advances in the treatment of erectile dysfunction.

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Characterization of the first microbial rhodopsin guanylyl cyclase (RhGC, same as BeCylOp) of *Blastocladiella emersonii,* generating cGMP.

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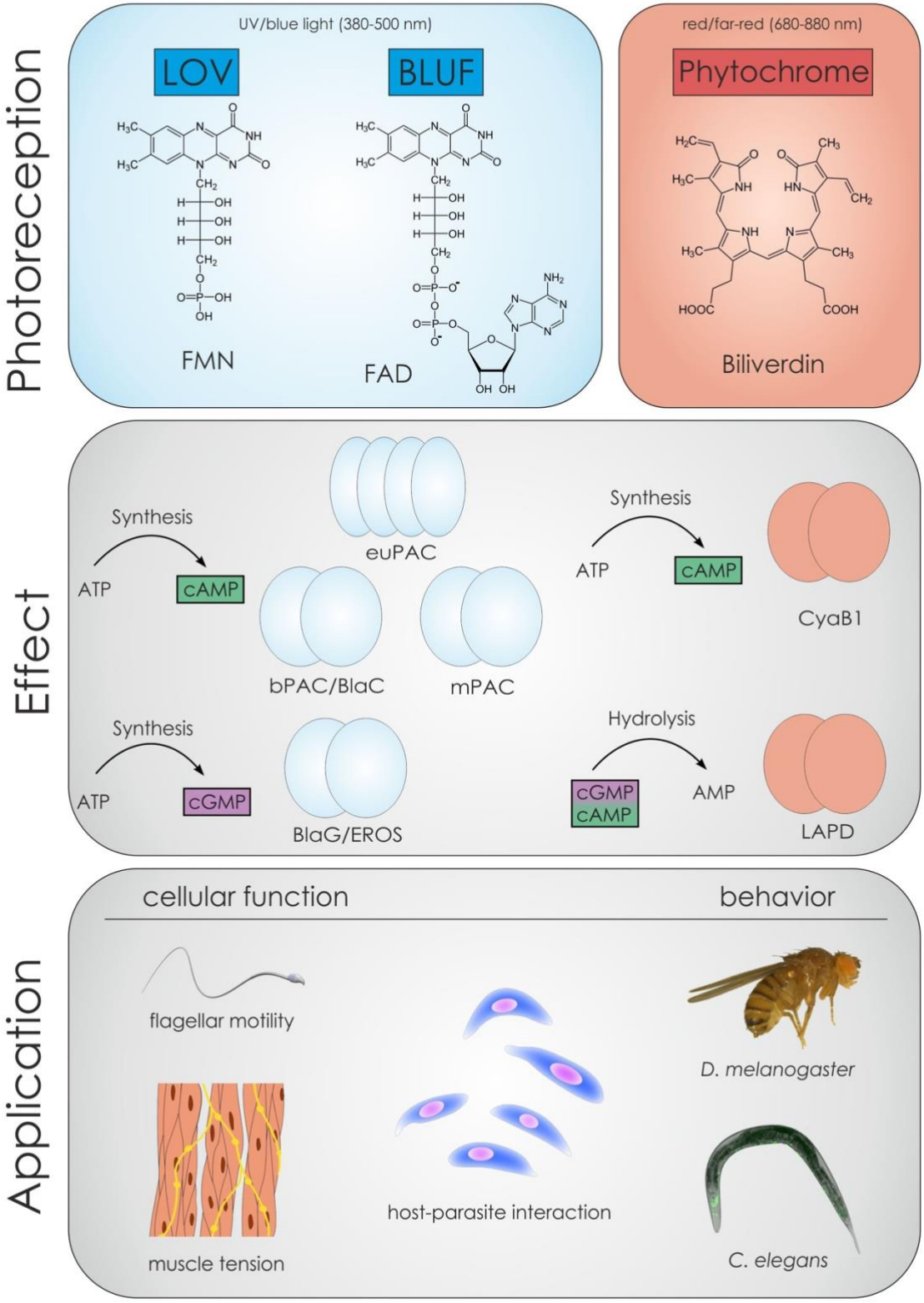
Generation of the first engineered light-activated phosphodiesterase (LAPD) by recombining the photosensory module of *Deinococcus radiodurans* bacterial phytochrome with the effector module of *Homo sapiens* phosphodiesterase 2A. LAPD mediated light-activated cAMP and cGMP hydrolysis in eukaryotic cell cultures and in zebrafish embryos and the biliverdin chromophore of LAPD is available endogenously and does not need to be provided exogenously.

Figure 1



Optogenetic tools carry a specialized domain for photoreception. Here, the incorporated cofactor of the photoreceptor domain (FMN/FAD/retinal/biliverdin) determines the wavelength of activation. Light-activated adenylate and guanylate cyclases synthesize cAMP and cGMP, respectively. So far, only one light-activated phosphodiesterase has been generated, which

degrades both cAMP and cGMP. Light-dependent modulation of cyclic nucleotides has been used to manipulate single cells, but also to change behavior of whole organisms.

Graphical abstract

An optogenetic tool is composed of a photosensor domain, which absorbs light, and an effector domain, which executes the enzymatic function. Optionally, a targeting sequence for restricted expression in subcellular compartments can be added. Local light stimulation allows to control the enzymatic activity with high temporal and spatial resolution.