Luminescence-activated nucleotide cyclase regulates spatial and temporal cAMP synthesis

Received for publication, July 16, 2018, and in revised form, December 12, 2018. Published, Papers in Press, December 17, 2018, DOI 10.1074/jbc.AC118.004905

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Edited by Henrik G. Dohlman

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This work was supported by National Institutes of Health NIDDK and NIGMS grants and the Molecular Pharmacology Training Program of the University of Pittsburgh under National Institutes of Health Awards R01-GM09975 (to D. L. A), R01-DK102495, R01-DK111427, and R01-DK16780 (to J. P. V.), National Institutes of Health Training Grants T32-GM008424 (to A. D. W.) and T32-GM0842419,20,21, and a Wistar Morris’ Cotswold Foundation Fellowship (to N. N.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This article contains Figs. S1–S9, Table S1, and Supporting Methods.

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‡ To whom correspondence should be addressed: Dept. of Pharmacology and Chemical Biology, School of Medicine, University of Pittsburgh under National Institutes of Health Awards R01-GM09975 (to D. L. A), R01-DK102495, R01-DK111427, and R01-DK16780 (to J. P. V.), National Institutes of Health Training Grants T32-GM008424 (to A. D. W.) and T32-GM0842419,20,21, and a Wistar Morris’ Cotswold Foundation Fellowship (to N. N.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Recent studies using Förster resonance energy transfer (FRET) (10–14), other biosensors, and mathematic modeling (15–18) have advanced the cAMP signaling paradigm by providing strong evidence that cAMP signaling requires tight spatio-temporal regulation. The resulting models propose that cAMP is regulated in distinct subcellular compartments containing adenylyl cyclases (ACs),4 cAMP effector proteins, phosphodiesterases, and scaffolding proteins. These regions create local areas of high and low cAMP concentrations that co-localize with cAMP effectors to regulate unique biological responses. Mounting evidence shows non-uniform cAMP accumulation throughout subcellular regions (i.e. plasma membrane versus bulk cytosol versus nucleus) (11, 13, 19–23). More recently, smaller scale compartmentalization or microdomains have been shown to exist and regulate cell function (10, 12, 13, 24–26). To identify bona fide compartments and define their function, new tools for localized cAMP synthesis are needed.

Distinct kinetic profiles characterize cAMP signaling. Transient cAMP production followed by signal termination is a classic pattern found in G protein– coupled receptor (GPCR) pathways (27–30). Sustained cAMP production can occur in several GPCR pathways (31–36) or from activating mutations that lead to constitutive cAMP synthesis (37–40). Finally, cAMP oscillations have been reported in pancreatic β, neuronal, pituitary, and myocardial cells (41–46).

Classically, cAMP levels are manipulated pharmacologically; however, drugs are usually slow (diffusion-limited) and non-targeted. Novel CAMP optogenetic tools were recently developed that offer precise temporal control over cAMP synthesis and targeting capabilities (47–53). However, despite its kinetic benefits, they require sophisticated optics and electronics for controlled stimulation. We reasoned that if coupled to a luminescence tool with proper spectral overlap, we could overcome the external cumbersome devices for a “systemic” intracellular delivery of light, as recently reported for channel rhodopsins.

cAMP is a ubiquitous second messenger that regulates cellular proliferation, differentiation, attachment, migration, and several other processes. It has become increasingly evident that tight regulation of cAMP accumulation and localization confers divergent yet specific signaling to downstream pathways. Currently, few tools are available that have sufficient spatial and temporal resolution to study location-biased cAMP signaling. Here, we introduce a new fusion protein consisting of a light-activated adenylyl cyclase (bPAC) and luciferase (nLuc). This construct allows dual activation of cAMP production through temporally precise photostimulation or chronic chemical stimulation that can be fine-tuned to mimic physiological levels and duration of cAMP synthesis to trigger downstream events. By targeting this construct to different compartments, we show that cAMP produced in the cytosol and nucleus stimulates proliferation in thyroid cells. The bPAC–nLuc fusion construct adds a new reagent to the available toolkit to study CAMP-regulated processes in living cells.

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4 The abbreviations used are: AC, adenylyl cyclase; sAC, soluble adenylyl cyclase; bPAC, photoactivated cyclase from Beggiatoa; nLuc, nanoluciferase; h-CIT, h-cocleterazine; Fz, furimazine; Fz-4377, endurazine prototype; NLS, nuclear localization signal, NES; nuclear export signal; ER, endoplasmic reticulum; MIYR, myristoylation; GPCR, G-protein– coupled receptor; LED, light-emitting diode; TSH, thyroid stimulating hormone; TSHR, TSH receptor; FBS, fetal bovine serum; H2O8, YFP–EPAC–Q270E–mScarlet; BrdU, 5-bromo-2’-deoxyuridine; EdU, 5-ethynyl-2’-deoxyuridine; IBMX, 3-isobutyl-1-methylxanthine; W, watt; YFP, yellow fluorescent protein; DAPI, 4’',6-diamidino-2-phenylindole.
Thus, using a similar strategy as the luminopsin design (54), we demonstrate that the fusion of photoactivated AC from Beggiatoa (bPAC) and nanoluciferase (nLuc) allows dual photo and chemical regulation of cAMP production for live-cell applications. Additionally, we have added a layer of spatial regulation using various targeting motifs to different subcellular compartments. We found cytosolic and nuclear cAMP is sufficient to trigger cell mitogenesis. Thus, the temporal and spatial flexibility of this construct makes it widely adaptable for in vitro and potentially in vivo applications.

**Results**

**Luminescence-activated cyclase allows photo and chemical activation of cAMP production**

To develop a tool with broad tunability, we prepared a fusion protein consisting of bPAC and nLuc. bPAC is a blue-light–sensitive cyclase with fast on/off kinetics and a high rate of synthesis (55). nLuc is a small (19 kDa) luciferase that emits blue-shifted luminescence in response to the luciferins furimazine (Fz) and h-coelenterazine (h-CTZ) (56). A Myc-tag was included to aid identification and visualization (Fig. 1A). In transfected HEK293 cells, h-CTZ elicits luminescence in the blue light range (maximum 455 nm) in a dose-dependent manner (mean ± S.D. of n = 4). D, h-CTZ increases cAMP in HC1 cells expressing bPAC–nLuc in a dose-dependent manner as shown by ELISA analysis (mean ± S.D. of n = 4). E, accumulation of cytosolic cAMP in HEK293 co-expressing H208 and MYR–bPAC–nLuc stimulated with pulses of 4.4 μW/mm² light (mean ± S.D. of n = 8 cells). F, stimulation with 1:1000 Fz promotes sustained cAMP production well over 30 min (mean ± S.D. of n = 4 cells). RLU, relative luminescence units.
bPAC–nLuc expression in HC1 cells shows similar luminescent properties as in HEK293 (Fig. S1, D and E).

The dual stimulation of cAMP production by bPAC–nLuc represents two signaling modalities: transient pulses of cAMP by light, and sustained cAMP production by luciferin stimulation. It is now well established that many GPCRs stimulate a transient peak of cAMP production from the plasma membrane and a following sustained phase of cAMP production during receptor endocytosis and redistribution in endosomes (31–33, 35, 59). Furthermore, multiple studies found oscillating patterns of cAMP production in response to pulses of blue light between 1 and 100 s at 4.4 \( \mu \text{W/mm}^2 \) (Fig. 1E), demonstrating bPAC retains its optogenetic properties in the fusion protein. cAMP levels returned to baseline values quickly, indicating tight temporal regulation with light. Fz induced a significantly longer signal, lasting well-beyond 30 min (Fig. 1F). Fz itself did not affect FRET ratio recordings (Fig. S4B). We found the duration and amplitude of cAMP levels were easily tuned by altering light intensity and duration or luciferin concentration and duration of exposure. Together, these two methods of stimulation can potentially tease out the individual effects of the transient, sustained, and oscillating temporal patterns of cAMP signaling.

**bPAC–nLuc stimulates cAMP-dependent thyroid cell proliferation**

Sustained cAMP production is beneficial for studying long-term processes such as cell proliferation. Using the PCCL3 rat thyroid cell model (60), which requires continuous thyroid-stimulating hormone (TSH)/cAMP signaling for S-phase entry (61–63), we examined the effects of light and chemical bPAC–nLuc activation. To find suitable conditions for light activation, we co-expressed membrane-localized bPAC–nLuc (MYR–bPAC–nLuc) and the cAMP FRET-based sensor, YFP–EPAC–Q270E–mScarletI (H208), in HEK293 cells. MYR–bPAC–nLuc generated transient spikes of cAMP accumulation in response to pulses of blue light between 1 and 100 s at 4.4 \( \mu \text{W/mm}^2 \) (Fig. 1E), demonstrating bPAC retains its optogenetic properties in the fusion protein. cAMP levels returned to baseline values quickly, indicating tight temporal regulation with light. Fz induced a significantly longer signal, lasting well-beyond 30 min (Fig. 1F). Fz itself did not affect FRET ratio recordings (Fig. S4B). We found the duration and amplitude of cAMP levels were easily tuned by altering light intensity and duration or luciferin concentration and duration of exposure. Together, these two methods of stimulation can potentially tease out the individual effects of the transient, sustained, and oscillating temporal patterns of cAMP signaling.

**Sustained cAMP signaling from localized bPAC–nLuc stimulates proliferation**

To investigate sustained, localized cAMP signaling, we incorporated different subcellular targeting motifs to bPAC–nLuc. bPAC and nLuc were initially selected for their small size, resulting in a final ~60-kDa fusion protein. Because it is just at the upper limit for passively diffusing through nuclear pores (65), we observed the unmodified construct evenly distributed throughout the cytosol and nucleus. By modifying the N and/or C termini with targeting motifs, bPAC–nLuc localization can be re-distributed to specific subcellular regions. In PCCL3, constructs localized to the nucleus (NLS), cytosol (NES), endoplasmic reticulum (ER) lumen, and membrane (CAAX and MYR, where A4 is aliphatic amino acid), as expected (Fig. 3A and Fig. S2D). All constructs luminesce immediately upon Fz stimulation, indicating effective drug access to the different compartments (Fig. S3A).

To monitor the behavior and efficacy of the targeted constructs, we modified the cytosolic H208 FRET sensor with an NLS sequence. The nuclear-targeted sensor (NLS-H208) exhibited similar cAMP affinity (EC_{50}). Hill coefficient, and range of activation in cell lyses (Fig. 4C). In transfected PCCL3 cells with similar levels of bPAC–nLuc expression, Fz-stimulated ER–bPAC–nLuc produced a small, transient
increase in cAMP in both the cytosolic and nuclear compartments. NES and NLS constructs produced greater amplitudes and overall cAMP levels comparable with TSH stimulation (Fig. 3B and Fig. S5). No significant difference between cytosolic and nuclear compartments was found under these conditions.

For sustained, chemical activation of bPAC–nLuc, Fz was specifically designed to activate nLuc providing a high signal and long duration (56). We found Fz-stimulated luminescence was still detectable after ~2 h (Fig. S3A). However, Fz was unable to induce cell proliferation in PCCL3 cells expressing bPAC–nLuc (data not shown). New Fz derivatives, such as endurazine and its prototype Fz-4377, have been developed to provide long-lasting luminescence and lower toxicity. Fz-4377 contains a protecting group that requires esterase hydrolysis for activity, resulting in low but stable luminescence for >6 h (Fig. S3B). PCCL3 cells stably expressing bPAC–nLuc triggered G1/S phase transition upon Fz-4377 treatment, indicating long-lasting luminescence-stimulated cAMP production is sufficient for a full proliferative response (Fig. 3C) at levels comparable (~30%) to 10 μM forskolin or 1 international milliunit/ml TSH stimulation (62, 66). NLS and NES bPAC–nLuc showed a similar increase; however, bPAC–nLuc localized to the ER lumen was unable to stimulate cell proliferation, despite its higher expression levels compared with the other constructs (Fig. S3C). Unlike the overexpression model, basal activity was not significantly increased. Thus, the combined results indicate that sustained cAMP synthesized in the cytosol or nucleus can stimulate thyroid cell proliferation.

**Discussion**

The cAMP field evolved from the original free diffusion model to a more complex model where cAMP amplitudes, kinetics, and subcellular location became critical elements to generate signaling specificity. Accordingly, sensors with greater sensitivity and dynamic range and actuators able to mimic different cAMP kinetic profiles were developed to test the new hypotheses. Optogenetic tools offer precise spatio-temporal resolution, and several new constructs developed were able to modulate cAMP levels in cells and animals. In this study, we report the characterization of bPAC–nLuc, a new reagent with dual photo and chemical stimulation, to study cAMP regulated processes in living cells.

Most studies approach the identification of cAMP compartments by measuring local cAMP levels with targeted FRET sensors or signalosome disruption (67, 68). However, causal association between a specific cAMP pool with a biological function requires reagents for localized cAMP synthesis. The soluble catalytic loops from G-protein–regulated cyclases, like the sACI/IIchimera, were used as a forskolin-sensitive tool to increase cAMP (69). Truncated soluble AC (sACt), activated by bicarbonate, has also been used to study the effects of membrane, cytosolic, and nuclear CAMP signaling (23). However, in both scenarios, activation also stimulates endogenous ACs. pH changes from bicarbonate can also interfere with fluorescent sensors and other cyclases (70, 71). Tsvetanova and von Zastrow (72) used targeted bPAC to show that cAMP produced at the plasma membrane or from endosomes yielded different transcriptional profiles. The temporal precision of bPAC is useful for transient activation of high CAMP concentrations; however, long-term stimulation can be limited by the hardware used to regulate light exposure (i.e. maintaining high voltage levels and overheating of LED) (73). To circumvent these current obstacles, the luminescence-activated cyclase presented
here offers the ease of chemical activation without activating endogenous cyclases and allows dual-light stimulation within the same construct to compare the effects of transient with sustained activation.

The bPAC–nLuc fusion protein is widely adaptable for experimental use. Both light and luciferin concentrations modulate cAMP synthesis in a predictable, tunable manner. bPAC–nLuc is also small in size and can be targeted to different compartments. Using a custom-built Arduino-controlled LED system (Fig. S6–S9 and Table S1), we can stimulate spikes of cAMP accumulation for seconds to minutes. With the advent of long-lasting Fz derivatives, chemical stimulation can promote luminescence for <1 h (h-CTZ) or beyond 6 h (Fz-4377). This combination presents a broad range of kinetic signatures to mimic different physiological cAMP signaling patterns within the same construct. A few disadvantages remain, including the need to minimize light exposure while working and high dark activity. In our thyroid cell model, overexpression leads to increased proliferation; fortunately, stable cell lines with lower expression exhibited lower basal proliferation. Hence, titrating expression with an inducible system may be recommended. The residual dark activity of bPAC has been noted by others (64) and must be considered when interpreting results.

Here, we demonstrate long-term luminescent stimulation of cAMP production can induce cAMP-dependent events, such as cell proliferation in thyroid epithelial cells. The thyroid represents a prototypical cAMP-responsive tissue where thyroid-stimulating hormone receptor (TSHR) signaling through cAMP is responsible for a majority of thyroid function (74). The combination bPAC–nLuc and long-lasting Fz-4377 stimulates proliferation similar to TSH-stimulated cAMP signaling (62, 66). bPAC–nLuc has the potential to test both the spatial (e.g., compartments involved) and temporal (e.g., along G0–G1/S) requirements of cAMP, which remain undefined in the field. cAMP synthesized in the ER lumen exhibited low cAMP levels reaching the nucleus and cytosol and was unable to stimulate proliferation. This may be a result of lower construct activity or reaching the nucleus and cytosol and was unable to stimulate cAMP synthesized in the ER lumen exhibited low cAMP levels. Although it is not explored here, bPAC–nLuc has many potential applications in vivo for target organs where LED implants would be too invasive. Transgenic mice expressing bPAC have been reported (78), and Fz has been used with success in mice to activate nLuc (79). Careful dosing is necessary to prevent toxicity (80), although new analogs for in vivo studies are being developed. The ability to synthetically stimulate localized cAMP signaling will play an important role, as the field of cAMP compartmentalization moves toward studying disease models and potential therapeutic interventions.

**Experimental procedures**

**Plasmid preparation**

Plasmids pcDNA3.1-bPAC-myc-mCherry (Addgene), pSirensRed, and myc-eV (Clontech) were obtained. bPAC-myc from pGEM-HE-hbPAC-cmyc (Addgene) and nLuc (Promega) were combined with several targeting sequences and subcloned in a pcDNA3.1+ backbone (Genescript). A series of restriction enzyme digests generated bPAC–nLuc (unmodified), ER–bPAC–nLuc (endoplasmic reticulum, 18-amino acid BiP signal sequence + AKDEL), and bPAC–nLuc–CAAX (farnesylated, KRas4B-CVIM). Additional N- and C-terminal targeting sequences were added to bPAC–nLuc (Cyagen Biosciences) to generate NLS–bPAC–nLuc (nuclear localization sequence, PKKKRKVEDA), and bPAC–nLuc–CAAX (farnesylated, KRas4B-CVIM). Additional N- and C-terminal targeting sequences were added to bPAC–nLuc (Cyagen Biosciences) to generate NLS–bPAC–nLuc (nuclear localization sequence, PPKKRKVEDA), NES–bPAC–nLuc (nuclear export sequence LQPLPLERLTL), and MYR–bPAC–nLuc (myristoylated/palmitylated, 17 amino acids N-terminal Yes). Lentinival constructs were made by subcloning bPAC–nLuc into the pCDH–puro-cmyc vector (Addgene, Cyagen Biosciences) and packaged (3rd generation, Cyagen Biosciences).

The cAMP FRET sensor H208 (YFP–EPAC–Q270E–mScarletI), kindly provided before publication by Dr. Jalink, consists of a YFP and mScarletI (81) fluorescent pair flanking catalytically inactive Epac1 with a mutation rendering high-affinity CAMP binding (82). It exhibits a loss in FRET signal upon binding cAMP. Note that the Jalink lab does not recommend this sensor for general experimentation due to punctate speckles forming a few days after transfection. This was confirmed in our hands but did not hamper our experiments, which were performed 2 days after transfection. Cells exhibited a relatively even cytosolic distribution of the sensor in the experiments shown here (Fig. S3A). For further details on the construct, please contact the Jalink laboratory. NLS-H208 was constructed using PCR to subclone an NLS motif to the N terminus by using HindIII restriction enzymes.

**Cell culture**

HC1 and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/liter), and streptomycin (100 mg/liter). PCCL3 cells were cultured as described previously (83). Stable cell lines were generated by lentiviral infection with a multiplicity of infection of 80 and 5 µg/ml Polybrene for 24 h and selected with puromycin. Transiently transfected cells were treated 24 h after plating with X-tremeGENE™ HP

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(Roche Applied Science) for HC1 cells or Lipofectamine 3000 (ThermoFisher Scientific) for PCCL3 and HEK293 cells.

Luminescence assays

3 \times 10^4 HEK293 were seeded in opaque 96-well dishes (Corning) coated with poly-d-lysine. 24 h after transfection, cells were washed with PBS, incubated with OptiMEM lacking phenol red, and stimulated with h-CTZ (NanoLight Technology) or NanoGlo® luciferase substrate (furimazine, Promega). The concentrations of Fz and its analogs are not provided; thus, we report treatments as dilutions from the stock solution. Luminescence was quantified using a Tecan Spark 20 M plate reader (SparkControl version 1.2) or SpectraMax Paradigm plate reader (SoftMax Pro 6.2.2) to measure specific wavelengths.

ELISA

1.5 \times 10^5 HCl cells were seeded in a 6-well dish, transfected, and stimulated for 10 min with 100 \mu M IBMX and h-CTZ. Cells were lysed and analyzed following the monoclonal anti-cAMP antibody-based direct ELISA kit (Neweast Biosciences) instructions and normalized by protein concentration using the Pierce™ BCA protein assay kit.

Optogenetic stimulation

Cells were kept in a dark environment using a red safelight lamp (Kodak GBX-2 Safelight Filter) with a 13-W amber compact fluorescence bulb (Low Blue Lights, Photonic Development LLC) to prevent light exposure from wavelengths <500 nm. Light activation was achieved using a custom-built, Arduino-controlled system capable of regulating the duration, frequency, and intensity of light exposure. The illuminating high-power LED (royal blue CREE XTE Tri-Star LED, LED Supply) was mounted on a stage (Fig. S5). The intensity range spanned from 4.41 ± 0.30 to 14.85 ± 1.00 \mu W/mm^2 as measured by a laser power meter (ThorLabs PM1100D, detector S130C) (Fig. 3B). For more details on the system, see Figs. S5–S8 and Table S1.

Real-time imaging

1.5 \times 10^6 PCCL3 cells seeded on 0.1% gelatin-coated 25-mm glass coverslips were transfected with a red dimerization-dependent sensor (Montana Molecular), H208, or NLS-H208. Cells were given fresh media for 24 h and hormone-starved for 3 h in Starvation Coon’s media (lacking TSH, insulin, and hydrocortisone) containing 5% FBS. Cells were washed in PBS and imaged in OptiMEM lacking phenol red on an Olympus IX70 microscope equipped with a Till Polychrome V monochromator. Images were acquired every 10 s with a \times 60/1.4 NA objective and with help from Dr. Watkins (Center for Biologic Imaging, University of Pittsburgh). Images were analyzed as % EdU-positive nuclei/total DAPI-stained nuclei using NIS Elements (Nikon Instruments). Immunofluorescent staining is included in the Supporting Methods.

Acknowledgments—We thank Dr. Kees Jalink (Netherlands Cancer Institute) for the H208 construct and Dr. Simon Watkins (Center for Biologic Imaging, University of Pittsburgh) for imaging support.

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