Improved molecular toolkit for cAMP studies in live cells

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Abstract

Background: cAMP is a ubiquitous second messenger involved in a wide spectrum of cellular processes including gene transcription, cell proliferation, and axonal pathfinding. Precise spatiotemporal manipulation and monitoring in live cells are crucial for investigation of cAMP-dependent pathways, but existing tools have several limitations.

Findings: We have improved the suitability of cAMP manipulating and monitoring tools for live cell imaging. We attached a red fluorescent tag to photoactivated adenylyl cyclase (PACα) that enables reliable visualization of this optogenetic tool for cAMP manipulation in target cells independently of its photoactivation. We show that replacement of CFP/YFP FRET pair with GFP/mCherry in the Epac2-camps FRET probe reduces photobleaching and stabilizes the noise level during imaging experiments.

Conclusions: The modifications of PACα and Epac2-camps enhance these tools for in vitro cAMP studies in cultured living cells and in vivo studies in live animals in a wide range of experiments, and particularly for long term time-lapse imaging.

Background

cAMP is a major cellular second messenger that activates and integrates multiple intracellular signaling pathways and modulates a large range of cellular processes, including gene transcription [1], cell adhesion and migration [2], and axonal growth and pathfinding [3]. cAMP studies rely on methods to manipulate and monitor cAMP concentrations in live cells. Existing tools have been very useful in identifying cAMP-dependent cellular processes, but have some limitations when it comes to understanding cAMP dynamics and localization in living cells. Forskolin and 3-isobutyl-1-methylxanthine (IBMX) are powerful pharmacological compounds enabling the generation of sustained elevations of cAMP. Forskolin directly stimulates most transmembrane adenylyl cyclases [4] and IBMX inhibits cAMP hydrolysis by phosphodiesterases. Recently, the use of photoactivated adenylyl cyclase alpha (PACα) from the flagellate Euglena gracilis, synthesizing CAMP in response to blue light, has allowed precise spatiotemporal manipulation of cAMP [5]. It has been attached to GFP for visualization in live cells [6]. However, the excitation wavelength of this visible reporter overlaps with the excitation spectrum of PACα, making it difficult to use this fusion construct for independent PACα excitation and reporter imaging.

Monitoring cAMP in live cells has been made possible by the use of FRET probes [7-10]. Epac2-camps is a cAMP indicator that is widely used to monitor cAMP [10] and has been recently improved with a mutation increasing its affinity for cAMP [11]. However, fast photobleaching of the commonly used CFP/YFP FRET pair limits its use in live cell imaging experiments over extended periods of time because the signal-to-noise ratio decreases progressively. The GFP/mCherry FRET pair has been successfully used for cAMP sensors [12], but its photostability and signal-to-noise ratio have not been assessed.

Results

Independent excitation of PACα and mCherry in living cells and live animals

The red fluorescent protein mCherry was expected to be an appropriate tag for PACα since its excitation wavelength in the green range of visible light [13] is distinct from PACα excitation by blue and UV light [14] (Figure 1A). A mCherry-PACα fusion protein was
generated using a mutant of PACα (R330A) that has a limited adenyl cyclase activity in the dark (G. Nagel, personal communication). mCherry was attached to the N-terminus of PACα (linker: SGLRSRAQASNSAVDGTA). The fluorescence of mCherry and light-dependent cAMP synthesis of PACα appear unaffected in the fusion product. mRNA coding for mCherry-PACα was transcribed using the mMessage Ultra kit (Ambion). 1 to 3 ng of mRNA were injected in both blastomeres of 2-cell stage Xenopus laevis embryos, which were incubated in the dark for 24 hr at 23°C. Dissociated cells from stage 21 embryo neural tubes were plated onto plastic dishes and kept in the dark for 2 hr. Cultures from injected but not control animals were fluorescent when illuminated at 561 nm on a Leica SP5 confocal microscope, revealing the expression of mCherry (Figure 1B). We then developed a bioassay to assess the function of this construct. Circus cells exhibit circular movement of the plasma membrane in cultures from Xenopus neural tubes [15]. Application of 10 μM forskolin blocked the circus movements of these cells (Figure 1B and 1C), demonstrating that a high concentration of cAMP prevents these plasma membrane movements. We next tested the light-dependent cyclase activity of mCherry-PACα by illuminating mCherry-positive cells at 488 nm. A 1-minute exposure abolished membrane movements. In contrast, mCherry excitation did not affect circus movements and
mCherry-negative cells did not exhibit plasma membrane movement arrest following stimulation by either wavelength (Figure 1B and 1C and see Additional file 1). We conclude that mCherry-PACα combines the features of mCherry for cell identification and PACα for light-sensitive cyclase activity, and that mCherry and PACα excitation wavelengths are exclusive from each other.

To verify that this construct can be used in vivo, *Xenopus* embryos injected with mCherry-PACα mRNA were illuminated with green and blue light in alternation using a fluorescence dissecting microscope with GFP and Texas-red filter cubes. Blue light illumination (excitation filter: BP 470/40) induced embryos to twitch, whereas they remain completely immobile under green light illumination (excitation filter: BP 560/40) (Figure 1D and 1E and see Additional file 2). Uninjected embryos did not exhibit light-induced twitching. Excitation of mCherry did not affect embryos’ behavior, confirming the spectral compatibility of mCherry and PACα.

**Reduced photobleaching of Epac2-camps using the GFP/mCherry FRET pair**

In addition to the improvement of PACα to manipulate cAMP, we modified the FRET pair of the existing Epac2-camps cAMP sensor to improve the stability of its signal-to-noise ratio over longer periods of time. We replaced the CFP/YFP pair by GFP/mCherry, with the same linkers as in the original probe [10]. mCherry and GFP have a low photobleaching rate [16] and constitute an efficient FRET pair [12] with smaller overlap between the emission spectra of the acceptor and the donor (Figure 2A). In addition, we included a mutation in the cAMP binding domain (K405E) that has been shown to reduce the Kd of the probe from 900 nM to 300 nM [11]. To limit cAMP synthesis locally. Further development of PACα may include its targeting to subcellular compartments to go beyond the limit of precision of optical stimulation and achieve cAMP manipulation bearing closer resemblance to physiological signals. It would be useful to ensure for each experimental condition that the cyclase activity of PACα in the dark does not affect intracellular signaling [5,17]. To limit cAMP synthesis without light exposure, we used a mutated PACα (R330A) that has a limited cyclase activity in the dark (G. Nagel, personal communication). This was enough to avoid perturbation of circuss cells movement by the cyclase activity of PACα in the dark. In case an extremely low dark activity is needed, bPAC, a bacterial light-sensitive adenylyl cyclase, could be used at the cost of less stringent temporal control of cAMP signaling [17].

pm-Epac2-camps-GFP/mCherry has greater photostability than pm-Epac2-camps-CFP/YFP and a lower noise level after extended periods of imaging. However GFP and mCherry make a less effective FRET pair than CFP and YFP, and its use may be beneficial only for FRET experiments requiring an extended period of imaging. Versions of CFP and YFP (mTurquoise and Venus respectively) with increased photostability are now available and make an efficient FRET pair for cAMP sensors [18]. Testing its noise level stability would allow comparison of the behaviour of GFP/mCherry and mTurquoise/Venus as FRET pairs for prolonged experiments. The
sensor described here has the advantage over the mTurquoise/Venus probe [18] of sensitivity to lower concentrations of cAMP, because it is an Epac2-based sensor including a mutation that reduces its Kd [11]. It would be useful to compare it to the Epac1-based sensor using mTurquoise/Venus, with the higher Kd for cAMP [1]. It would further improve the shift of PAC switch of the mCherry tag to a longer wavelength fluorescent tag and the FRET acceptor.

The use of both tools in the same cell is not yet possible due to the overlap of excitation wavelengths, but further improvements may include the shift of PACα excitation towards the UV to avoid wavelength conflict with the FRET probe excitation, in combination with the switch of the mCherry tag to a longer wavelength fluorescent protein such as mKate to avoid the overlap of emission between the PACα tag and the FRET acceptor.

Additional material

Additional file 1: Photoactivation of PACα mimics forskolin-induced arrest of circus cells in culture. In circus cells in cultures from the X. laevis neural tube, blebs of plasma membrane propagate around the cell circumference with a period of several minutes [Olson, 1996]. Forskolin stimulation of endogenous adenylyl cyclases in control cells arrests this blebbing movement (top left panel). Blue light (488 nm) irradiation of mCherry-PACα-expressing cells mimics the effect of forskolin stimulation (top right panel). In contrast, blebbing movements are not affected in non-blue light-irradiated mCherry-PACα-expressing cells (bottom left panel) or in control cells illuminated at 488 nm (bottom right panel).

Additional file 2: Photoactivation of PACα induces X. laevis embryo twitching. mCherry-PACα-injected embryos twitch when illuminated with blue light to excite PACα, but not when exposed to green light to excite mCherry.

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Authors’ contributions
KPH and XN carried out the experiments. NCS and XN conceived the experimental design. KPH, NCS and XN wrote the manuscript. All authors have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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