

Fast manipulation of cellular cAMP level by light *in vivo*

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The flagellate *Euglena gracilis* contains a photoactivated adenylyl cyclase (PAC), consisting of the flavoproteins PAC α and PAC β . Here we report functional expression of PACs in *Xenopus laevis* oocytes, HEK293 cells and in *Drosophila melanogaster*, where neuronal expression yields light-induced changes in behavior. The activity of PACs is strongly and reversibly enhanced by blue light, providing a powerful tool for light-induced manipulation of cAMP in animal cells.

cAMP is a ubiquitous second messenger across phyla¹ and multiple adenylyl cyclases, and phosphodiesterases are involved in its formation and degradation, respectively. A light-activated adenylyl cyclase that is crucial for photoavoidance has been identified in the unicellular flagellate *Euglena gracilis*². This adenylyl cyclase is composed of two PAC α and two PAC β subunits, which exhibit adenylyl cyclase activity that is enhanced by blue light. Each subunit harbors two BLUF-type photoreceptor domains, binding flavin adenine dinucleotide^{3,4}, and two catalytic domains that are homologous to class III adenylyl cyclases². Until now, it was unclear whether the individual subunits are catalytically active and whether they are active in animal cells.

Manipulation of cellular signaling in live animals with the help of genetically encoded light-sensitive proteins has become feasible in recent years^{5–7}. Expression of a light-sensitive adenylyl cyclase in cells would allow the manipulation of cAMP with exquisite spatiotemporal control. To this end, we functionally expressed PACs (encoded by PAC α and PAC β) in two popular expression systems, *X. laevis* oocytes and HEK293 cells. Moreover, transgenic *D. melanogaster* flies demonstrated functional PAC expression by showing blue light-induced behavioral changes.

We expressed c-myc-tagged PAC α and PAC β in *Xenopus* oocytes and detected products of ~110 kDa and ~90 kDa in PAC α - and

PAC β -expressing oocytes, in good agreement with the respective molecular weight of PAC α (112 kDa) and PAC β (94 kDa; **Supplementary Fig. 1** online and **Supplementary Methods** online). We determined the total intracellular concentration of cAMP ([cAMP]_i) in single oocytes by an immunoassay (**Fig. 1a** and **Supplementary Data** online). Control oocytes had a mean [cAMP]_i of 1.3 ± 0.6 μM. PAC α -expressing oocytes displayed a pronounced adenylyl-cyclase activity at rest. After 4 d of expression in the dark, [cAMP]_i was ~20-fold enhanced compared to controls. When we irradiated PAC α -expressing oocytes by blue light for 5 min, [cAMP]_i increased further about tenfold, demonstrating light-stimulated adenylyl cyclase activity. We emphasize that the high [cAMP]_i values obtained with the immunoassay refer to total [cAMP]_i (much of it bound to cAMP-binding proteins). Control and PAC β -expressing oocytes displayed no substantial changes in [cAMP]_i upon blue-light irradiation for 5 min (**Fig. 1a** and **Supplementary Data**). The [cAMP]_i of PAC β -expressing oocytes was not substantially different from that of control oocytes.

We used the human cystic fibrosis transmembrane conductance regulator⁸ (CFTR) as a cAMP sensor to monitor light-induced changes in [cAMP]_i. CFTR is a Cl⁻ channel that is activated by phosphorylation via cAMP-dependent protein kinase (PKA)⁹. We coexpressed CFTR with PAC α , PAC β , or PAC α and PAC β in oocytes. The high basal activity of PAC α required us to reduce the expression level by injecting only ~200 pg of cRNA. Application of the phosphodiesterase inhibitor, IBMX, and the activator of endogenous adenylyl cyclase, forskolin, caused a large increase in membrane conductance (**Fig. 1b**), as has been previously shown for cells that express CFTR only¹⁰. A short pulse of blue light mimicked this pharmacological effect by causing a similar increase in conductance after a delay of 15–20 s (**Fig. 1b**). The amplitude of the response increased with the intensity or duration of light stimulation (**Supplementary Fig. 2** online). For strong irradiation, the delay of the electrical response was as short as 2 s (**Fig. 1c**).

When we injected ~200 pg of PAC β cRNA into oocytes, we observed no light-induced change in conductance. When we injected 20 ng of PAC β cRNA, however, irradiation of oocytes with blue light caused an increase in conductance (**Fig. 1b**) similar to that of oocytes injected with 200 pg of PAC α cRNA. The results of experiments in which PACs were coexpressed with CFTR in oocytes are summarized in **Figure 1d**.

To examine the kinetics of the light-induced cAMP production, we coexpressed PAC α or PAC β with cyclic nucleotide-gated (CNG) channels. CNG channels are directly opened by cAMP and cGMP, and mutant CNG channels with different sensitivities are available. For the study of PAC α , we selected the mutant CNGA2-T537S

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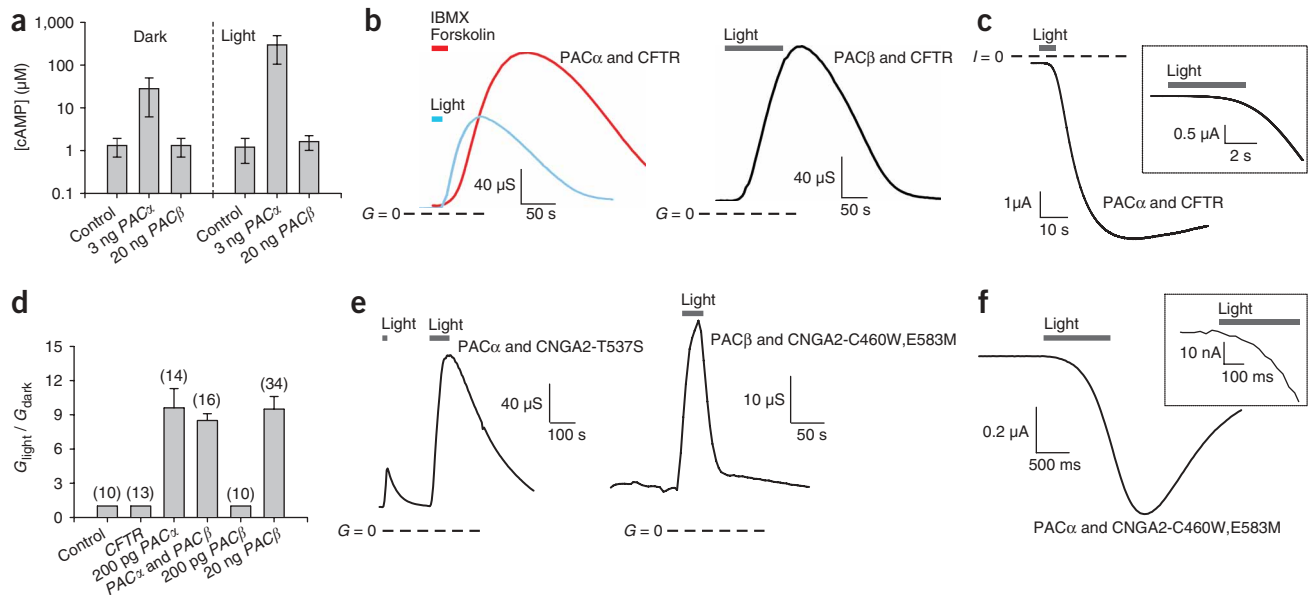


Figure 1 | Expression of PACs in oocytes. **(a)** Total [cAMP], 4 d after injection of cRNA. Oocytes were kept in the dark or irradiated for 5 min with blue light (380–480 nm). Control, noninjected oocytes. Error bars, mean \pm s.d. **(b)** Oocytes expressing CFTR and PAC α (200 pg cRNA, left) or PAC β (20 ng cRNA, right). Bars indicate duration of drug application (red) or light pulse (blue, black). Conductance (G) is plotted against time. We applied 0.5 mM IBMX, 10 μ M forskolin to a PAC α and CFTR-expressing oocyte (red trace), and the same oocyte was irradiated with blue light (blue trace; left). **(c)** CFTR current upon high-intensity irradiation (from a light-emitting diode with 28 mmole photons $m^{-2} s^{-1}$) of a PAC α -expressing oocyte. **(d)** Summary of light-induced conductance changes when expressing PACs with CFTR. Control = not injected. The amount of PAC-cRNA injected and the number of experiments is indicated below and above the bars, respectively. Student's t -test: at the 0.01 level, the difference of the population means is significant for control and PAC α , for control and 20 ng PAC β , and for control and PAC α -PAC β ; the difference of the population means is not significant for control and CFTR, for control and 200 pg PAC β , for 200 pg PAC α and 20 ng PAC β , and for PAC α and PAC α -PAC β . **(e)** Coexpression of PAC α with CNGA2-T537S (left), of PAC β with CNGA2-C460W,E583M (right). Light pulses indicated by bars. **(f)** Photoactivated inward current at -60 mV of an oocyte expressing PAC α with CNGA2-C460W,E583M.

(*Bos taurus* CNGA2), which is characterized by a constant of half-maximal activation, K_{cAMP} , of 14 μ M (ref. 11). Coexpression of PAC α and CNGA2-T537S gave rise to a fast and reversible light-induced increase of conductance (Fig. 1e). The light-induced activity of PAC β was not sufficient to activate the CNGA2-T537S channel (data not shown). Therefore, we coexpressed PAC β with a more sensitive mutant CNGA2-C460W,E583M ($K_{cAMP} = 1$ μ M; ref. 12; *Rattus norvegicus* CNGA2). Oocytes, expressing PAC β and CNGA2-C460W,E583M, showed a fast and reversible light response (Fig. 1e). We also expressed PAC α with the CNGA2-C460W,E583M channel to estimate the rate of PAC α activation. The photocurrent increased almost instantaneously after the onset of irradiation (Fig. 1f). We estimate the time constant for the activation of PAC α to be ≤ 20 ms, that is, the time resolution of the recordings. We also found that light-dependent activity of PAC α switches off within a few milliseconds after light has been switched off (Supplementary Fig. 2), confirming and extending results on purified PAC α -PAC β ¹³. It was not possible to switch off PAC by applying light of different wavelengths to specifically excite a photocycle intermediate. This agrees with a minimal spectral shift (10 nm to the red) of the BLUF signaling state^{3,4}.

To examine the suitability of PACs as a tool to manipulate [cAMP]_i, we expressed PAC α in HEK293 cells with CNGA2-T537M as a cAMP sensor ($K_{cAMP} = 3$ μ M). We monitored the activity of the CNG channel by its Ca²⁺ permeability using the fluorescent Ca²⁺ indicator Fluo-4. We used the same excitation light ($\lambda_{exc} = 480 \pm 10$ nm) to stimulate PAC α and Fluo-4. When we increased the light intensity tenfold, control cells only displayed a

step increase of Fluo-4 fluorescence (Fig. 2a), whereas PAC α -expressing cells displayed an additional slower increase in fluorescence, indicating light-stimulated Ca²⁺ influx through CNG channels (Fig. 2b). To monitor Ca²⁺ without strongly stimulating PAC α , we used a photoshutter to produce short flashes of light to measure Fluo-4 fluorescence. The Ca²⁺ signal reached a plateau upon continuous light stimulation; when we closed the photoshutter and probed the fluorescence every 2 s with a short flash of light, the Ca²⁺ signal declined to a baseline level within about 30 s, reflecting phosphodiesterase activity of the cell (Fig. 2c). In the presence of IBMX (100 μ M), the light-stimulated Ca²⁺ response was similar, but the signal declined only slightly. Finally, we investigated the resting activity of PAC α by monitoring the fluorescence of Fluo-4 without continuous illumination (Fig. 2d). Upon addition of IBMX to the bath, the fluorescence increased slowly and reached a stable plateau after ~ 200 s. We observed no such increase in control cells that expressed the CNGA2-T537M channel only (data not shown).

To test the potential of transgenic PAC expression *in vivo*, we used the Gal4-UAS enhancer expression system¹⁴ for targeting of PACs to the *D. melanogaster* brain. Neuronal expression of either PAC α or PAC β with the *elav*-Gal4 driver line resulted in adult flies without any obvious behavioral phenotype. Irradiating adult flies with blue light resulted in periods of hyperactivity and unusual freezing behavior in flies expressing PAC α (Supplementary Video 1 online), but not in wild-type flies (Supplementary Video 2 online) or in flies expressing PAC β , even under the control of the strong *tubulin* promoter (*tub*-Gal4/UAS-PAC β ; Supplementary

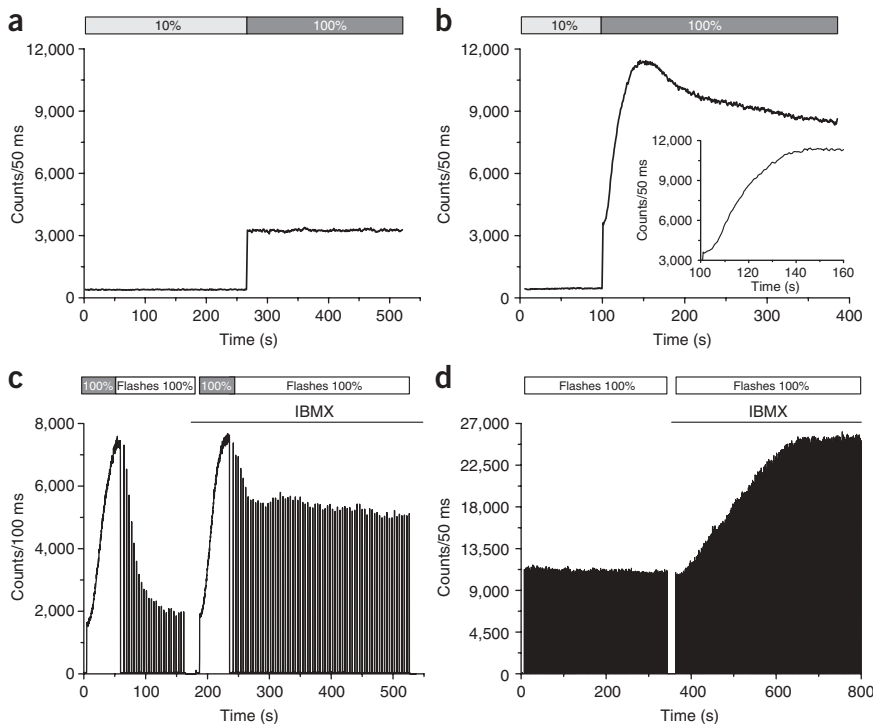


Figure 2 | Expression of PAC α in HEK293 cells. (a) Fluorescence of a CNGA2-T537M cell loaded with Fluo-4AM. A gray filter, blocking 90% of light, was removed from the light path at the indicated time to increase excitation light intensity. (b) Same experiment as in a but with a CNGA2-T537M cell expressing PAC α . Inset, the rising phase of the light response on an expanded time scale. (c) Time course of the increase and decrease of fluorescence of a PAC α and CNGA2-T537M cell. After activation of PAC α with continuous light (gray bar), the decline of fluorescence was monitored by 200 ms flashes of light every 2 s (white bar). IBMX (100 μ M) was added to the bath solution as indicated. (d) Time course of fluorescence of a PAC α and CNGA2-T537M cell. The fluorescence was monitored every 2 s by 200 ms flashes of light (bar). No continuous light stimulation was used. IBMX (100 μ M) was added to the bath as indicated. 100% light = ~ 250 W/m 2 = ~ 950 μ mole photons m $^{-2}$ s $^{-1}$.

In this study, we have successfully expressed the PAC α and PAC β from the flagellate *E. gracilis* in multiple systems where they functioned as light-sensitive adenylyl cyclases, albeit with different enzymatic activities. We conclude that

enzymatic turnover, that is, light-activated cAMP production, of PAC α is about 100-fold that of PAC β . Notably, we demonstrate that the free cAMP concentration produced by light stimulation is sufficient to activate important targets for cAMP, namely cAMP-dependent protein kinase (PKA) and CNG channels. This tool may provide exquisite spatiotemporal control of cAMP levels in future work of signaling pathways in transgenic models, particularly for tackling questions of learning and memory in *Drosophila*.

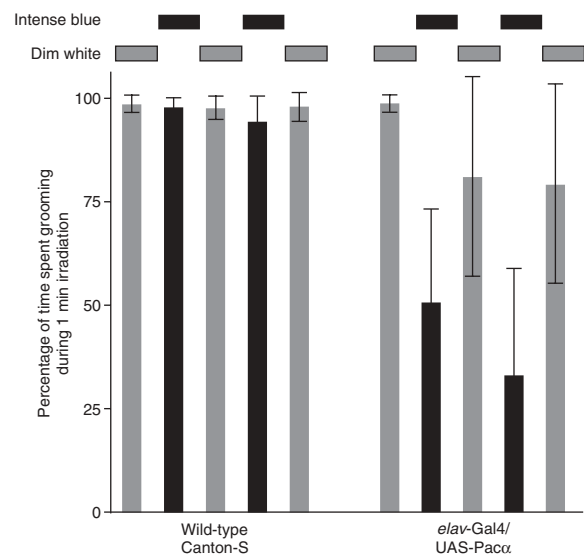
Advantages of this new method are: (i) the adenylyl cyclase is genetically encoded by a single gene; (ii) no chemical modification of the protein is required; (iii) no addition of chromophore is needed; (iv) the substrate (ATP) is plentiful; and (v) the chromophore or expressed protein is not toxic if expression is limited.

Fig. 3 online). Similarly, irradiation of *elav-Gal4-UAS-PAC α* flies with the same light intensity but different λ_{exc} (590 \pm 10 nm) did not affect behavior (**Supplementary Video 3** online).

To determine the reliance and kinetics of the light-induced change in behavior, we analyzed the grooming reflex¹⁵. When covered with a fine powder, fruit flies instantaneously display vigorous and continuous grooming activity lasting up to 30 min (data not shown). Monitoring this behavior for a total time of 5 min with irradiation alternating between dim white light (from a cold light source) and intense blue light (from a light-emitting diode, λ_{exc} = 455 \pm 10 nm) for 1 min each, revealed high grooming activity in wild-type Canton-S flies irrespective of stimulation by light (**Fig. 3**). In contrast, neuronal expression of PAC α (*elav-Gal4/UAS-PAC α*) resulted in hyperactivity and a substantial decline in grooming activity under blue-light stimulation. When irradiation was switched back to dim white light, thus turning off blue light-induced PAC activity, flies returned to grooming behavior within several seconds (**Supplementary Video 1** and **Supplementary Table 1** online). These results demonstrate that transgenic expression of PAC α in fruit flies results in a functional protein that is rapidly and reversibly activated by blue light. Moreover, the fast action observed at the on- and offset of irradiation demonstrates the feasibility of rapid control of cAMP levels in a freely moving animal.

Figure 3 | Photostimulation of PAC α alters behavior in freely moving *D. melanogaster*. Grooming activity of individual flies was scored in five consecutive intervals of 1 min each with irradiation switching between either dim white light (for observation purposes) or intense blue light of 455 nm for PAC activation. Irradiation conditions are shown above the bars. Statistical analysis was performed to identify light-induced changes within each genotype using Wilcoxon signed ranks test (wild type, $P > 0.05$; *elav-Gal4-UAS-PAC α* , $P < 0.001$ when comparing blue and dim white light conditions). Data represent means \pm s.d. ($n = 9$) except for *elav-Gal4-UAS-PAC α* ($n = 33$).

Figure 3 shows a bar graph of grooming activity. The y-axis is 'Percentage of time spent grooming during 1 min irradiation' ranging from 0 to 100. The x-axis shows two genotypes: Wild-type Canton-S and *elav-Gal4/UAS-Pacα*. For each genotype, there are four bars representing different light conditions: Dim white, Intense blue, Dim white, Intense blue. Above the bars, gray bars indicate 'Dim white' and black bars indicate 'Intense blue' irradiation. For Wild-type Canton-S, grooming activity is high (around 90-100%) in both white and blue light. For *elav-Gal4/UAS-Pacα*, grooming activity is high in white light but significantly lower (around 30-50%) in blue light.



Compared to photo-uncaging, this method is advantageous as no precursor is exhausted, it is easier to use, and it faces no complications with diffusion or premature degradation of the caged compound.

The basal activity of PAC α may be a disadvantage for specific applications, but there are alternatives: weak expression of PAC α or expression of the 100-fold less active PAC β . Future work on PACs might allow suppression of resting activity by appropriate mutation(s), thereby further improving a powerful tool for manipulating [cAMP] by light.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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