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cAMP_r: A single-wavelength fluorescent sensor for cyclic AMP[#]

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Abstract

Genetically encoded fluorescent sensors enable cell-specific measurements of ions and small molecules in real time. Cyclic adenosine monophosphate (cAMP) is one of the most important signaling molecules in virtually all cell types and organisms. Here, we describe cAMP_r, a new single-wavelength cAMP sensor. We developed cAMP_r in bacteria and embryonic stem cells and validated the sensor in mammalian neurons in vitro and in *Drosophila* circadian pacemaker neurons in intact brains. Comparison with other single-wavelength cAMP sensors showed that cAMP_r provided improved quantitative detection of cAMP abundance. In addition, cAMP_r is compatible with both single-photon and two-photon imaging. This enabled us to use cAMP_r together with the red fluorescent Ca²⁺ sensor RCaMP1h to simultaneously monitor Ca²⁺ and cAMP in *Drosophila* brains. Thus, cAMP_r is a new and versatile genetically encoded cAMP sensor.

Introduction

Live imaging is a powerful tool to understand the dynamics of cell signaling, because it enables constant monitoring of an individual cell over time (1). Numerous sensors are available that report real-time changes in the amounts of small molecules and ions or the activities of specific biochemical pathways. Genetically encoded sensors are useful, because they can be targeted to specific cell types within tissues and even to subcellular regions. Most genetically encoded sensors have either one or two fluorescent proteins fused to one or two protein domains. These protein domains alter conformation in response to changes in small-molecule or ion concentrations. Single fluorescent protein sensors typically use

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switches in conformational states of the fused protein(s) to alter the chromophore environment of the fluorescent protein and change the amount of fluorescence. Sensors with two fluorescent proteins link conformational changes of the fused protein domain to changes in the distance between the two fluorescent proteins, which can be monitored by changes in Förster resonance energy transfer (FRET) or fluorescence lifetime imaging (FLIM).

The genetically encoded sensor GCaMP is a single-wavelength Ca^{2+} sensor (2). GCaMP consists of a circularly permuted green fluorescent protein (cpGFP) with the M13 peptide from myosin light chain kinase fused to the N-terminus of cpGFP and calmodulin (CaM) fused to the C-terminus. CaM interacts with M13 in a Ca^{2+} -dependent manner, which leads to increased cpGFP fluorescence that reflects intracellular Ca^{2+} . GCaMP has gone through multiple rounds of design over 15 years, culminating with GCaMP6, which is sensitive enough to record single action potentials, shows comparable performance to small-molecule Ca^{2+} indicators, and can be used to map neuronal circuits (3, 4).

Cyclic adenosine monophosphate (cAMP) is a widely used second messenger in living organisms. The amount of cAMP differs between cells, and increasing cAMP in different subcellular compartments can result in different outputs (5, 6). Many neurotransmitters and neuropeptides activate heterotrimeric guanine nucleotide protein (G protein)-coupled receptors (GPCRs) that regulate adenylate cyclase activity and either increase or decrease the concentration of intracellular cAMP. Changes in cAMP concentration have numerous effects in neurons, including regulating gene expression through the transcription factor CREB (7–9). Because changes in cAMP concentration can be locally triggered at synapses, it may be possible to monitor the strength and location of synaptic connections by detecting changes in cAMP abundance.

Multiple genetically encoded cAMP sensors are available, but the ones with the best kinetics, intensity, and fold change are based on Epac-1 and Epac-2 (Exchange protein directly activated by cAMP). Epac proteins are guanine nucleotide exchange factors (GEFs) with a cAMP-binding domain; GEF activity depends on cAMP binding. Epac-based cAMP sensors use FRET between CFP and YFP, in which CFP is excited and emissions from CFP and YFP are monitored. Increasing the amount of cAMP decreases FRET, leading to an increase in CFP emission and a decrease in YFP emission (10, 11). These FRET-based Epac-cAMP sensors have also undergone several rounds of improvements, mostly by switching the fluorescent proteins fused to Epac-1 or Epac-2 (12, 13). Other cAMP sensors include FRET sensors based on Protein Kinase A (PKA). However, these sensors respond more slowly and with a smaller fold change than Epac-based sensors and require transfecting cells with two separate constructs, which limits their usefulness (11). AKARs are another family of FRET sensors, although these detect the activity of PKA signaling pathways rather than changes in cAMP (14).

Although these sensors have helped the understanding of cAMP signaling, CFP/YFP FRET imaging has some drawbacks. For example, the shorter wavelengths required to excite CFP tend to penetrate tissues poorly, making FRET sensors more difficult to image. Other limitations of FRET include the differential scattering of photons from CFP and YFP, the tendency for CFP and YFP to bleach, and CFP also emitting fluorescence in the YFP

channel (15). In addition, simultaneously measuring cAMP and Ca^{2+} in a single neuron is desirable, but this is challenging with a FRET sensor that already uses two fluorescent proteins. A single-wavelength sensor for cAMP (cADDIs) based on Epac-2 was developed, but it remains to be fully characterized in vivo and is only available commercially (16). Although this sensor is a step forward for cAMP imaging, there are still substantial issues to be resolved. cADDIs has only been tested in mammalian cell culture, and it is unknown if cADDIs adversely affects cellular physiology. cADDIs also has a relatively high K_d for cAMP (~10 to 100 μM) in vitro; thus, this sensor may only detect relatively high amounts of cAMP. To combat the limitations of the various FRET and Epac-based sensors, we developed a new single-wavelength sensor for cAMP.

We constructed a sensor using PKA, because the cAMP-dependent protein interactions of PKA have been extensively studied (17–19). The strategy that we used was a rapid preliminary screen in bacteria followed by transfecting embryonic stem (ES) cells with constructs encoding the brightest variants. With this second step, we tested sensor variants in rapidly dividing cells and then in post-mitotic neurons derived from ES cells. We called the resulting sensor cAMP_r. We tested cAMP_r in circadian pacemaker neurons in intact *Drosophila* brains and demonstrated that cAMP_r responds robustly to endogenous signals within an intact neural circuit. Finally, we showed that cAMP_r can be used with RCaMP1h, a red-shifted fluorescent Ca^{2+} reporter, to simultaneously monitor cAMP and Ca^{2+} in individual neurons in the intact fly brain. Thus, cAMP_r is a new and versatile genetically-encoded sensor for cAMP.

Results

Rationale for using PKA-R amino acid residues 91 to 244

PKA exists as a holoenzyme of two catalytic (PKA-C) and two regulatory (PKA-R) subunits. PKA-R subunits dimerize through the Dimerization/Docking (D/D) domain, which can also direct the subcellular localization of the PKA holoenzyme. We used amino acid residues 91 to 244 of PKA-R, which lack the D/D domain, to ensure that the sensor would remain in the cytoplasm (19). Removing the D/D domain changes the overall stoichiometry of the PKA-R: PKA-C complex to 1:1 and should also prevent interaction with endogenous PKA subunits. Full-length PKA-R has two cAMP-binding domains, which function cooperatively. However, the second cAMP-binding domain is missing in PKA-R residues 91 to 244 (19). This increases the in vitro K_d for cAMP from 10 nM for full-length PKA-R to 1 μM and reduces the cooperativity of cAMP binding from $n = 1.5$ to $n = 1$ (19). Together, these properties should extend the range of a sensor based on PKA-R residues 91 to 244, which is important because cAMP concentrations span a wide range in different cell types (20).

Initial sensor construction and linker library screening

We initially constructed two versions of the sensor. C-G-R consists of PKA-C fused to the N-terminal of cpGFP with a Pro-Gly linker and PKA-R residues 91 to 244 fused to the C-terminal of cpGFP with an Ala-Cys linker (Fig. 1A). R-G-C had the opposite configuration

of PKA subunits relative to that of cpGFP and lacked linkers. R-G-C fluoresced in bacteria but showed no response to forskolin in ES cells and thus was not pursued.

We expressed C-G-R in *E. coli* where it was targeted to the periplasm. The first C-G-R construct with the shortest linkers did not fluoresce in *E. coli* at either 18 or 37°C. Because amino-acid linkers can affect both basal fluorescence and the fold-change of cpGFP-based sensors (2, 21), we constructed linker libraries consisting of ~30,000 individual clones (Fig. 1B). Many bacteria fluoresced under the microscope, albeit most quite weakly. However, the fluorescence occurred in inclusion bodies, and we could not detect any change in fluorescence upon adding cAMP. Thus, we selected the brightest variants by flow cytometry and then moved to a eukaryotic system to test their responsiveness to cAMP. We isolated 8 bacteria that were ~5- to 10-fold brighter than most of the ~1,000,000 bacteria that were sorted.

Isolating forskolin-responsive cAMP sensors in ES cells

We chose ES cells to characterize cAMP sensor variants for two reasons. First, site-specific recombination can be used to integrate DNA into a defined genomic location (22). This should remove variation in expression due to chromosomal location, thereby facilitating comparison between clones expressing different variants. Second, ES cells can be differentiated into numerous cell types, enabling a cAMP sensor to be tested in different cellular environments, including neurons.

The variants from the 8 bacteria selected by flow cytometry were cloned as a pool into the P2Lox vector (22) (Fig. 2A). A17iCRE ES cells were transfected and DNA integration was selected by antibiotic resistance. We induced sensor expression by adding doxycycline for 2 days. Imaging revealed a mix of fluorescent and non-fluorescent colonies. We then applied forskolin to stimulate adenylate cyclase activity to increase the concentration of intracellular cAMP. Adding forskolin increased the fluorescence of the fluorescent colonies by ~30 to 40%; no change was seen in the non-fluorescent colonies (Fig. 2B). Adding the vehicle DMSO did not change the fluorescence (Fig. 2B).

To identify the genotype of the forskolin-responsive clones, we selected 6 fluorescent clones. Sequencing revealed that 4 of the 6 fluorescent clones had the original Pro-Gly sequence as the N-terminal linker and Thr-Ala-Pro-Pro-Ala-Cys as the C-terminal linker. Surprisingly, the other 2 clones had the original Pro-Gly and Ala-Cys linkers as N- and C-terminal linkers, respectively (Fig. 2A). There were no mutations in either variant. To determine which variant should be fully characterized, we directly compared the performance of the two forskolin-responsive variants. Cells from 2 clones of each variant were plated in 24-well plates and imaged before and after the addition of 40 μ M forskolin. The two variants had similar fold-changes in fluorescence and kinetics (fig. S1). We selected the sensor with the Trp-Ala-Pro-Pro-Ala-Cys C-terminal linker, because it had a slightly faster response time to forskolin than the shorter linker variant and because it fluoresced in both ES cells and bacteria. We named this variant cAMP_r.

ES cells expressing cAMP_r exhibited a ~50% increase over baseline fluorescence in response to forskolin and did not exhibit a response to DMSO (Fig. 2B, movies S1 and S2).

Furthermore, cAMP_r had a cytosolic distribution in ES cells before and after exposure to forskolin or DMSO, as predicted from deleting the PKA-R D/D domain (19). The excitation and emission spectra for cAMP_r expressed in ES cells with and without forskolin had the expected excitation and emission maxima for GFP (fig. S2). Thus, increasing the cAMP concentration with forskolin only changed the fluorescence output of cAMP_r and not its excitation or emission profiles.

Specificity of cAMP_r for cAMP in ES cells

For cAMP_r to be a genuine cAMP sensor, the increase in fluorescence with forskolin must be due to increases in cAMP abundance, not secondary or indirect changes in other cyclic nucleotides. To test this, we used digitonin to permeabilize the membranes of ES cells expressing cAMP_r and exposed them to either 1 mM cAMP or 1 mM cGMP. cAMP_r showed a robust ~40% increase in fluorescence in response to cAMP but no change in response to cGMP (Fig. 2C). Thus, cAMP_r exhibited specificity for cAMP over cGMP and directly responded to changes in cAMP concentration.

Comparison of cAMP_r to cADDis in ES cells

Next, we compared the performances of cAMP_r and cADDis. To obtain ES cells expressing cADDis, A17iCRE ES cells were infected with a baculovirus expressing cADDis. Because this produced cells with a range of fluorescence intensities, we chose cells with a similar fluorescence to that of cAMP_r-expressing ES cells. We plated the cells in 24-well plates and imaged them two days later. We compared the average normalized response to decreasing amounts of forskolin for ES cells expressing cAMP_r or cADDis (Fig. 2D). The data showed that cAMP_r had a more linear response to different forskolin concentrations, whereas cADDis had a more sigmoidal response (Fig. 2E). Although cAMP_r had a slightly lower maximum fold-change than that of cADDis, we concluded that cAMP_r was a better sensor for detecting small changes in cAMP concentration.

Kinetics and imaging of cAMP_r in ES cells

Next, we tested how accurately cAMP_r fluorescence intensity reflected changes in cAMP concentration in real time by measuring cAMP_r responses to transient forskolin application. To dynamically control cAMP amounts, we plated ES cells on glass coverslips and performed the imaging in a flow chamber. ES cells expressing cAMP_r showed no response to DMSO applied for 180 s (Fig. 3A, blue bar), whereas 40 μ M forskolin (Fig. 3A, red bar) increased fluorescence by ~25%. The fluorescence gradually dissipated back to baseline by ~9 min. This timeframe is consistent with the expected rates of cAMP hydrolysis, suggesting that cAMP_r fluorescence intensity mirrored the changes in cAMP concentration (23).

To test whether cAMP_r retained sensitivity over multiple stimulations, we sequentially applied 40 μ M forskolin to ES cells expressing cAMP_r in a flow chamber after the fluorescence values returned to baseline. The responses to the first and second applications of forskolin were similar both in fold-change and kinetics (Fig. 3B), indicating that cAMP_r can undergo more than one activation cycle without losing sensitivity. Because two-photon imaging enables better sample penetration than single-photon imaging, we tested whether

cAMPr was compatible with two-photon imaging and thus could be useful for deep tissue imaging. We excited ES cells expressing cAMPr in a flow chamber with a two-photon laser at 920 nm and exposed the cells to forskolin. We found that cAMPr produced a similar fold-change and kinetic profile to forskolin under two-photon (Fig. 3C) and single-photon excitation (Fig. 3, A and B). Thus, cAMPr is compatible with two-photon imaging and should be useful for in vivo applications.

Testing cAMPr in mammalian neurons

ES cells can be differentiated into different cell types. Because we were particularly interested in using cAMPr to study neuronal signaling, we differentiated ES cells expressing cAMPr into neurons. We induced ES cells to form embryoid bodies (EBs) and then added either retinoic acid (RA) or a Smoothed agonist (SAG) to differentiate the cells into two different populations of neurons (Fig. 4A) (24, 25). RA-induced differentiation produces a mixture of different types of ventral spinal cord neuron progenitors (24), whereas activation of Hedgehog signaling pathways directs ES cells to become ventral forebrain neurons (25). To test whether cAMPr functioned in mammalian neurons, we measured the response of RA-differentiated and SAG-differentiated neurons to forskolin. Both RA-differentiated neurons (Fig. 4B and movie S3) and SAG-differentiated neurons (Fig. 4C, movie S4) responded to forskolin with an increase in fluorescence. Next, we assessed whether cAMPr could detect changes in cAMP stimulated by endogenous GPCRs. For this, we treated the differentiated neurons with dopamine, which can either increase or decrease cAMP concentration, and GABA, which either decreases cAMP or activates a chloride channel. Most of the SAG-differentiated neurons (>95%) responded to dopamine with increased fluorescence (Fig. 4C, movie S5). The response was specific for dopamine, because GABA did not cause any change in fluorescence (Fig. 4C).

RA-differentiated neurons expressing cAMPr also responded to dopamine, but not to GABA (fig. S3, A and B, movie S6). However, the responses were more variable than those of SAG-differentiated neurons, which is consistent with the diversity of neurons resulting from RA differentiation protocols (24). The results from experiments with neurons derived from ES cells expressing cAMPr indicate that this cAMP sensor is sufficiently sensitive to detect neurotransmitter signaling mediated by endogenous GPCRs in cultured neurons. We exposed SAG-differentiated neurons expressing cAMPr to increasing concentrations of dopamine and calculated an effective concentration (EC50) of these neurons for dopamine of ~5 μ M (fig. S3C), which is similar to the reported Kd of 2.5 to 4 μ M of the dopamine receptor D1 for dopamine (26, 27). We also performed a similar experiment with SAG-differentiated neurons infected with cADDIs-expressing baculovirus (Fig. 4D, fig S3D). In contrast to the cAMPr-expressing neurons, the cADDIs-expressing neurons appeared to give an all-or-none response to decreasing amounts of dopamine, whereas cAMPr showed a more sigmoidal response (Fig. 4D). Thus, cAMPr produced a more accurate quantitative response to dopamine concentrations than did cADDIs in neurons differentiated from ES cells.

Testing cAMPr in *Drosophila* pacemaker neurons

To test cAMPr in an intact brain, we chose to study *Drosophila* circadian pacemaker neurons. These neurons respond to the Pigment Dispersing Factor (PDF) neuropeptide by

increasing the concentration of cAMP, and they were previously used to validate Epac-camps in transgenic flies (28). We generated transgenic flies by integrating *UAS-cAMPr* into a defined genomic locus using the attB/attP targeting system.

We first focused on the lateral ventral circadian pacemaker neurons (LN_vs). There are two types of LN_vs in adult flies: the small LN_vs (s-LN_vs) are the principal pacemaker cells during constant darkness (29), whereas the large LN_vs (l-LN_vs) are not critical for maintaining circadian rhythms, but are more important in sleep (30, 31). Using the *Pdf-Gal4* promoter, we expressed cAMPr in all adult LN_vs. To test whether cAMPr detected changes in cAMP abundance induced by forskolin in the whole brain, we dissected brains from adult flies, placed the brains in a glass-bottom dish, and used two-photon imaging to measure fluorescence in the LN_vs in response to either 40 μ M forskolin or solvent (DMSO). We quantified the change in fluorescence from both sets of LN_vs, which exhibited an ~40% increase over baseline in cAMPr fluorescence in response to forskolin and a small decrease in fluorescence in response to DMSO (Fig. 5A). Thus, cAMPr responded to forskolin in an intact brain and detected changes in cAMP abundance in a non-mammalian system.

We compared cAMPr to the FRET-based cAMP sensor Epac-camps, which we expressed in LN_vs using the *Pdf-Gal4* promoter. Forskolin stimulated a ~35% decrease in the YFP:CFP ratio due to the decrease in FRET (Fig. 5B, data are shown inverted for easy comparison to Fig. 5A) in the dissected adult brains, which is similar to previous reports (28); DMSO had no effect. The data showed that cAMPr and Epac-camps responded to forskolin with similar fold-changes and kinetics. Thus, cAMPr resolves the limited dynamic range and slow kinetics of the previous PKA-based sensors and does not have the limitations of FRET-based sensors with two fluorescent proteins (11, 14).

Because cAMPr contains an active kinase and because genetically encoded sensors can disrupt cellular physiology, we tested whether LN_vs that expressed cAMPr functioned normally. This is particularly important because altering PKA activity or cAMP abundance can change the period length and strength of *Drosophila* locomotor activity rhythms in constant darkness (32–34). However, flies expressing *UAS-cAMPr* controlled by *Pdf-Gal4* in LN_vs had strong rhythms and a period length of 23.8 hours in constant darkness, which was not statistically significantly different from those of heterozygous *UAS-cAMPr* flies (23.7 hours) or *Pdf-Gal4* control flies (23.9 hours) (table S2). These data contrast with those obtained from LN_vs expressing *UAS-Epac-camps*, which exhibit a period lengthened by ~1 hour (28), suggesting that Epac-camps alters either the amount of cAMP or cAMP signaling pathways in LN_vs. Thus, cAMPr seems to interfere less with cAMP signaling pathways in LN_vs than does Epac-camps.

We next tested whether cAMPr was sensitive enough to detect signaling in a neuronal circuit within the brain by stimulating LN_vs to fire and then measuring the cAMP responses of downstream neurons. We used the larval circadian neural network, which is simpler than the adult clock network and has only 9 clock neurons per hemisphere (35). These 9 neurons can be divided into subgroups: 5 LN_vs project to the dorsal brain and communicate with 4 dorsal neurons (DNs); 4 of the 5 LN_vs release the neuropeptide PDF, which increases intracellular cAMP concentration by binding to and activating the G α s-coupled PDF Receptor (PDFR),

which is likely found in all larval clock neurons (28, 36). We expressed cAMP_r in all larval clock neurons using *tim-Gal4*. To induce LN_v firing and thus PDF release, we also expressed the mammalian purinergic receptor P2X₂ in LN_vs using a *LexAOp-P2X₂* transgene (Fig. 5C). ATP activates P2X₂ receptors, which depolarizes neurons, causing them to release neurotransmitters and neuropeptides. Because there is no P2X₂ ortholog in *Drosophila*, adding ATP to brains with *Pdf-LexA* and *LexAOp-P2X₂* specifically activates LN_vs.

We performed two-photon imaging of larval brains with P2X₂ expressed only in LN_vs and cAMP_r in all clock neurons and exposed the brains to 2.5 mM ATP. We found that applying ATP increased cAMP_r fluorescence in DN_s (Fig. 5D, movie S7). These responses required P2X₂ expression in LN_vs because cAMP_r fluorescence in DN_s from control larvae without a P2X₂ transgene decreased slightly after the addition of ATP (Fig. 5D). However, all neurons imaged from these control brains responded to forskolin, indicating that cAMP_r was functional in DN_s (fig. S4). Analogous experiments with GCaMP in adult s-LN_vs also showed slightly decreased fluorescence after the addition of ATP to brains lacking P2X₂, suggesting that this may be a general response of GFP derivatives to ATP (37, 38).

Larval LN_vs expressing both cAMP_r and P2X₂ also responded to ATP with increased cAMP_r fluorescence (Fig. 5E and movie S8). This was expected because adult s-LN_vs express PDFR and respond to exogenously applied PDF (28). However, equivalent experiments in adult s-LN_vs using Epac-camps did not detect changes in cAMP after s-LN_v depolarization (37). This difference probably results from the increased sensitivity of cAMP_r compared with that of Epac-camps at detecting small changes in cAMP concentration. Thus, our data from experiments with *Drosophila* circadian pacemaker neurons show that cAMP_r can detect endogenous signals in the whole brain as assessed by two-photon imaging and suggest that cAMP_r is more sensitive than Epac-camps for detecting cAMP signaling in vivo.

Simultaneously measuring Ca²⁺ and cAMP in whole brains

Ca²⁺ is another key signaling mediator of neuronal signaling; thus, it would be helpful to be able to simultaneously measure changes in cAMP and Ca²⁺ concentrations. As a single-wavelength reporter, cAMP_r should make this possible when used in conjunction with RCaMP1h, a red-shifted, genetically encoded Ca²⁺ sensor (21). We used *Pdf-Gal4* to simultaneously express UAS transgenes encoding cAMP_r and RCaMP1h in larval LN_vs. Larval brains were dissected and then treated with carbachol, a general cholinergic agonist. Carbachol depolarizes PDF-expressing LN_vs (34, 39), which increases intracellular Ca²⁺ concentrations through voltage-gated Ca²⁺ channels and leads to the release of PDF from the LN_vs. PDF then activates PDFR on LN_vs in an autocrine or paracrine manner, which increases the concentration of cAMP in LN_vs. We monitored RCaMP1h and cAMP_r fluorescence in LN_v cell bodies and projections simultaneously (Fig. 6A). We noted that RCaMP1h fluorescence increased in LN_v projections before it increased in cell bodies (Fig. 6B and movie S9). The fold-change in cAMP_r fluorescence was greater in LN_v cell bodies than in the projections, which may indicate differences in PDF release, PDFR density, or signaling strength (Fig. 6C). We also noted that the increase in Ca²⁺ preceded the increase in cAMP in cell bodies and projections (Fig. 6D and E).

The previous experiment used single-photon imaging. We also tested whether cAMP and Ca^{2+} could be simultaneously monitored by two-photon imaging (fig. S5). Two-photon (fig. S5A) and single-photon (Fig. 6D) imaging of LN_ν s expressing the two sensors and exposed to carbachol produced similar changes in fluorescence patterns, although the fold-changes were lower with two-photon excitation. We monitored the fluorescence of both sensors with two-photon excitation in isolated larval brains exposed to forskolin instead of carbachol (fig. S5B). As expected, forskolin stimulated an increase in cAMP α fluorescence without affecting RCaMP1h fluorescence. We conclude that cAMP α is a versatile genetically encoded cAMP sensor, which should be widely useful in neurobiology and beyond.

Discussion

We have generated a new single-color cAMP reporter that is compatible with two-photon imaging and seems to minimally perturb cellular physiology. Tests in both mouse ES cells and in neurons differentiated from ES cells showed ~50% increases in cAMP α fluorescence after appropriate stimulation and a return to baseline fluorescence in ~10 minutes, which is consistent with the kinetics of cAMP signaling. Testing cAMP α in circadian pacemaker neurons in *Drosophila* brains revealed that cAMP α exhibits a fold-change similar to that of the existing Epac family of sensors and is compatible with non-mammalian systems. We also compared cAMP α to the only other available single-wavelength cAMP sensor, cADDi, and showed that cAMP α gave more quantitative readouts of cAMP amounts in both ES cells and neurons. Finally, we showed that cAMP α can be used to detect signaling within an endogenous neural circuit and to simultaneously measure Ca^{2+} and cAMP in *Drosophila* brains.

Although functional in its current version, we believe that cAMP α can be improved in the future. For example, the affinity of cAMP α for cAMP could be altered by incorporating previously studied point mutations in the PKA regulatory subunit (40). This could shift the dynamic range of cAMP α to monitor higher or lower cAMP concentrations, which might be necessary for using cAMP α in other cell types or in subcellular compartments with different cAMP amounts from those tested here. Our current version of cAMP α is cytoplasmic, but it could also be targeted to specific subcellular compartments.

In summary, we showed that cAMP α is functional and has minimal effects on cellular physiology in both mammalian cell culture and *Drosophila* brains. In addition, cAMP α is more sensitive to small changes in cAMP abundance than cADDi. cAMP α can also be easily combined with other single-wavelength sensors, unlike Epac-camps. Finally, cAMP α showed robust responses using either single- or two-photon imaging, with the latter being particularly important for experiments involving either deep tissue or live animals. We believe that cAMP α possesses many of the characteristics to make it the sensor of choice to study cAMP signaling in a range of different tissues and organisms.

Materials and methods

Constructing C-G-R and R-G-C

The cDNAs encoding PKA-C, PKA-R (91–244), and cpGFP were amplified from the plasmids pET15b PKA Cat (41), pRSETB PKA R1 α (42), and TorPE-G-GECO1.2 (43) (Addgene plasmids 14921, 14922, and 32468, respectively) using Phusion High-Fidelity DNA Polymerase (NEB). Purified PCR products were mixed together in 1:2:3 ratios (PKA-C:cpGFP:PKA-R) and amplified for 10 cycles without primers. Primers were added and the DNA was amplified for a further 20 cycles. The sequences encoding C-G-R and R-G-C were subcloned into the pTorPE vector (digested with Xba I and Hind III) using T4 DNA ligase (Promega) and the resulting constructs used to transform bacteria. Clones were selected and their sequences verified. The primers used are listed in table S2.

Constructing and sorting C-G-R linker libraries in bacteria

C-G-R libraries were constructed by digesting pTorPE_C-G-R with SphI and XmaI. We amplified cpGFP with the appropriate linker library primers for each pool. PCR products were purified, digested with SphI and XmaI, and ligated into pTorPE_C-G-R that had been digested with the same enzymes. Bacteria were transformed with the contents of the ligation reaction and 5 plates were plated per library, resulting in ~30,000 individual clones per library. All three libraries were scraped, mixed together, and grown overnight at 37°C with 0.001% w/v Arabinose (Sigma) to induce expression. Cells were sorted for GFP expression on a FACS Aria flow cytometer. Eight bright fluorescent outliers were sorted into LB containing ampicillin and grown overnight at 37°C before undergoing DNA extraction.

Generation of ES cell lines

The brightest C-G-R clones from bacteria were amplified and subcloned into Eco RI–digested p2Lox vector (22) using In-Fusion cloning (Clontech). Bacteria containing the C-G-R mini-library were scraped off a plate and grown in LB containing ampicillin before DNA extraction. A2Lox.cre ES cells (22) were then transformed with the purified DNA using a standard nucleofection protocol (Lonza).

C-G-R mini-library imaging and clone selection in ES cells

After transformation, ES cells containing C-G-R were cultured until they were ~80% confluent. Cells were trypsinized, plated at low density on a 35-mm dish, and grown for 5 to 6 days until colonies reached a sufficient size for imaging. Colonies were imaged on a Nikon Eclipse Ti microscope before and after the application of forskolin. Six different clones showing increased fluorescence after forskolin addition were picked and expanded. DNA was extracted for sequencing using a standard phenol/chloroform genomic DNA extraction. Each clone was reimaged before and after forskolin application to ensure a correct match between fluorescent phenotype and DNA sequence.

Differentiating ES cells into neurons

ES cells were differentiated using standard embryoid body formation followed by treatment with either 1 μ M RA or 500 nM SAG (24, 25). Differentiated neurons were maintained in

motor neuron buffer composed of Neurobasal, 2% fetal bovine serum (FBS), $1 \times$ B27, 500 nM l-glutamine, 10 nM beta-mercaptoethanol, 4 mM 2'-deoxy-5-fluorouridine, and 4 mM uridine (44). Doxycycline (3 mg/ml) was added to induce cAMP_r expression.

Imaging cAMP_r in ES cells

ES cells were imaged in either 24-well plates or a flow chamber. For 24-well plates, cells were seeded in wells treated with 0.1 w/v gelatin and then cultured in medium containing doxycycline (3 mg/ml) until they reached 25 to 50% confluency. Cells were washed three times with $1 \times$ Hanks' Balanced Salt Solution and imaged on a Leica SP8 confocal microscope every 60 s. Either 40 μ M Forskolin or DMSO was applied at the times indicated in the figure legends for the initial characterization experiments. For cAMP and cGMP experiments, 1 μ M digitonin was applied at the beginning of imaging, and either 1 mM cAMP or cGMP was added at the times indicated in the figure legends. For flow chamber experiments, cells were seeded as described earlier except that a glass coverslip was used. The glass coverslip was placed in a 100-mm cell culture dish and the cells were cultured as described earlier. Cells were then imaged every 60 s with either single or two-photon excitation on an Olympus FLV 1000, with drugs added at the times indicated by gray shaded rectangles in the graphs. We used standard GFP filter sets for single-photon excitation. For two-photon imaging, laser excitation was set at 920 nm with standard filter sets used for monitoring emission.

Imaging cAMP_r in differentiated neurons

Differentiated neurons were plated in 24-well plates pre-coated with 0.01% Poly-L-Lysine and imaged as described for ES cells, except that images were captured every 80 s. Neurons were treated with 40 μ M Forskolin, DMSO, 100 μ M GABA, or 100 nM to 1 mM dopamine at the times indicated in the figure legend.

Generating *UAS-cAMP_r* flies

The cDNA encoding cAMP_r was inserted into Eco RI-digested pUASattB using In-Fusion cloning (Clontech). The pUASattB-cAMP_r construct was injected into the VK37 landing site on chromosome 2 by Rainbow Transgenics. Four independent *UAS-cAMP_r* fly lines were tested for their expression of cAMP_r in LN_vs by crossing to *Pdf-Gal4* flies. All lines exhibited the correct spatial expression and one line was used for imaging.

Imaging in *Drosophila* brains

Pdf-Gal4 (45) and *tim(UAS)-Gal4* (46) flies were crossed to either *UAS-cAMP_r; Pdf-LexA*, *LexAOP-P2X₂* or *UAS-Epac-camps; Pdf-LexA*, *LexAOP-P2X₂* flies. Recombinant *Pdf-LexA*, *LexAOP-P2X₂* flies were kindly provided by Orie Shafer (University of Michigan). Adult flies were anesthetized on ice and dissected in HL3 media. Whole brains were imaged in glass bottom dishes pretreated with 0.001% Poly-L-lysine (Sigma) in HL3 medium at room temperature. An Olympus-FLV1000 microscope was used with 488 nm excitation and standard GFP emission settings for cAMP_r or with 445 nm excitation and standard CFP/YFP FRET emission settings for Epac-camps. ATP (100 mM, Sigma) was dissolved in 20 mM Tris-HCl and the pH was adjusted to 7.2.

Simultaneously measuring Ca^{2+} and cAMP in whole brains

Pdf-Gal4 flies were crossed with *UAS-cAMP_r* flies to generate a *UAS-cAMP_r; Pdf-Gal4* line. These flies were then crossed with *UAS-RCaMP1h* flies (21) to generate flies expressing cAMP_r and RCaMP1h in the PDF-expressing LN_vs. Flies were dissected and imaged as described earlier except that a Leica SP8 microscope was used. Excitation at 488 nm and 561 nm was used to image cAMP_r and RCaMP1h, respectively. Two-photon imaging was performed with an Olympus FLV 1000 using 920 nm excitation.

Image quantification

All images were quantified with Fiji/Image J software. For ES cells, regions of interest (ROIs) generally included whole colonies, although only a portion of the colony was used for larger colonies. When imaging ES cells that had differentiated into neurons, we only quantified the soma. If two or more cell bodies were directly adjacent, they were quantified together using a single ROI. All values were background-subtracted. The fluorescence at a particular time was divided by the fluorescence at time zero to generate the change in fluorescence values plotted in the graphs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Editor's summary**Live-cell imaging of cAMP**

The second messenger cAMP is found in many cell types and organisms. Increases in the cytosolic concentration and localization of cAMP mediate various cellular functions through the activation of the kinase PKA and the transcription factor CREB; thus, the ability to detect and image cAMP in live cells would help to understand its mechanism of action. Hackley *et al.* developed a genetically encoded fluorescent sensor of cAMP (cAMPr), which was expressed in mammalian neurons and in the *Drosophila* brain and responded to stimulation of adenylyl cyclase, the enzyme that generates cAMP. Furthermore, cAMPr was used together with a fluorescent Ca^{2+} sensor to simultaneously detect cAMP and Ca^{2+} in the *Drosophila* brain through two-photon imaging, suggesting its potential use to study cAMP signaling in diverse tissues and organisms.

Fig. 1. Design and selection of a cpGFP-based cAMP sensor

(**A**) Schematic and putative mechanism of cAMPr. PKA-C (orange) is the full-length catalytic subunit of PKA; cpGFP (green) is circularly permuted GFP; and PKA-R 91–244 (blue) contains amino acid residues 91 to 244 of the regulatory subunit of PKA. The binding of cAMP (yellow) to the PKA-R peptide triggers a conformational change that releases PKA-C from PKA-R and increases fluorescence. (**B**) Flow chart of linker library generation and screening as a TorA fusion in *E. coli* (gray). PG is a two-amino acid linker inserted between PKA-C (C: orange) and cpGFP (G: green). AC is a two-amino acid linker inserted between cpGFP and PKA-R amino acids 91–244 (R: blue) to generate C-G-R. Three

different linker libraries were made with 4 random amino acids (X)₄ inserted between PG and cpGFP (left), or between PG and cpGFP and between cpGFP and AC (middle), or between cpGFP and AC (right). The most strongly fluorescent bacteria were selected by flow cytometry analysis. The chosen bacteria were then grown before plasmid DNA was extracted.

Fig. 2. Initial testing and characterization of cAMPr in ES cells

(A) Overview of the screening process in ES cells. Forskolin was added to ES cells expressing the cAMP sensor variants and those colonies that exhibited an increase in fluorescence were selected and sequenced. The sequences of the linkers of the forskolin-responsive clones are shown. (B) Left: Response of ES cell colonies expressing cAMPr to 40 μM forskolin (red, n = 15 colonies) or DMSO vehicle (blue, n = 16 colonies) from two independent experiments. The gray box indicates the time of drug addition. Light red and blue shading represents the SEM. Data are normalized to the fluorescence at time zero. Right: Images show representative single ES colonies before (left) and after (right) the

application of 40 μ M forskolin (top) or DMSO (bottom). *P* was calculated using a standard unpaired two-sided *t* test. (C) Response of cAMP_r in digitonin-permeabilized ES cells to 1 mM cAMP or 1 mM cGMP. Left: Graph shows the average of at least 13 colonies from two independent experiments and the data were plotted as described in (B). Right: Bar graph showing the maximum fold-changes in fluorescence plotted for 1 mM cAMP (red) and 1 mM cGMP (Blue). (D) Response of ES cell colonies expressing cAMP_r (left) or cADDIS (right) to the indicated concentrations of forskolin. Cells expressing cADDIS that were exposed to DMSO are shown in black. Data are from 14 to 20 colonies from two independent experiments for all treatments. Data are plotted as described in (B). (E) Maximum average fold-change in fluorescence in response to the indicated concentrations of forskolin in ES cells expressing cAMP_r (left) or cADDIS (right) normalized to the maximum response to 40 μ M forskolin using data from (D). Two-sample unpaired *t*-tests for cAMP_r showed *P* < 0.05 for each value plotted compared to the next highest concentration (40 μ M vs. 10 μ M: *P* < 0.05; 10 μ M vs. 1 μ M: *P* < 0.001; 1 μ M vs. 100 nM: *P* < 0.001; 100 nM vs. 10 nM: *P* < 10⁻⁵). For cADDIS, two-sample unpaired *t* tests only showed statistical significant differences for 1 μ M vs 100 nM when compared to the next highest value (40 μ M vs. 10 μ M: *P* = 0.7; 10 vs. 1 μ M: *P* = 0.5; 1 μ M vs. 100 nM: *P* < 10⁻⁸, 100 vs. 10 nM: *P* = 0.10; 10 nM vs. DMSO: *P* = 0.44).

Fig. 3. cAMPr kinetics and two-photon compatibility in ES cells in a flow chamber

(A) Response of cAMPr-expressing ES cells to DMSO (blue) and 40 μ M forskolin (red) applied for the indicated periods to cells in a flow chamber. Data are plotted as the fold-change in fluorescence normalized to that at time zero. Graph shows an average of 10 colonies from a single experiment imaged by single-photon excitation. (B) Response of cAMPr-expressing ES cells to sequential exposure to forskolin. 40 μ M forskolin (red) was applied to cAMPr-expressing ES cells in a flow chamber from $t = 120$ s to 240 s and from $t = 660$ s to 800 s. The graph shows an average of 15 colonies from a single experiment using single-photon excitation and the data are plotted as described in (A). (C) Response of

cAMPr-expressing ES cells to 40 μ M forskolin (red) in a flow chamber and imaged by two-photon microscopy. Graph shows an average of 15 colonies from a single experiment and the data are plotted as described in (A).

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Fig. 4. cAMP responses in ES cells induced to differentiate into neurons

(A) Schematic of the protocols used to induce ES cells to differentiate into neurons. Doxycycline was added to induce cAMP expression. (B) Left: Response of RA-differentiated neurons expressing cAMP to either 40 μ M forskolin (red) or DMSO (blue). Data are from 14 and 18 neurons, respectively, from two independent experiments. Data are plotted as described in Fig. 2B. Right: Images of individual representative RA-differentiated neurons expressing cAMP showing fluorescence before (Pre) and after (Post) the addition of 40 μ M forskolin (top) or DMSO (bottom). (C) Left: Response of SAG-differentiated neurons expressing cAMP to 10 μ M forskolin, 1 mM dopamine, or 100 μ M GABA. Data

are from 18 to 20 neurons per treatment, with two replicates per treatment, from two independent experiments. Data are plotted as described in Fig. 2B. Right: Representative images of a typical SAG-differentiated neuron expressing cAMPr before (Pre) and after (Post) the addition of 1 mM dopamine. The same neuron is shown in grayscale (top) and pseudocolor (bottom). (D) Responses of SAG-differentiated neurons expressing either cAMPr (left) or cADDIs (right) to the indicated concentrations of dopamine. Fluorescence was quantified from 15 to 20 neurons for each dopamine concentration from two independent experiments. Two-sample unpaired *t* tests showed statistical significance of $P < 0.05$ for all dopamine concentrations in neurons expressing cAMPr except for 1 mM vs. 100 μ M when compared to the next highest value (1 mM vs. 100 μ M: $P = 0.90$, 100 μ M vs. 10 μ M: $P < 0.05$; 10 μ M vs. 1 μ M: $P < 10^{-8}$; 1 μ M vs. 100 nM: $P < 0.01$). For cADDIs-expressing neurons, only 10 μ M vs. 1 μ M was statistically significantly different (1 mM vs. 100 μ M: $P = 0.07$; 100 μ M vs. 10 μ M: $P = 0.90$; 10 μ M vs. 1 μ M: $P < 10^{-7}$; 1 μ M vs. 100 nM: $P = 0.75$).

Fig. 5. Testing cAMP in *Drosophila* pacemaker neurons

(A) Top: Diagram showing an adult *Drosophila* brain and the approximate location of adult LN_vs expressing cAMP in *Pdf-Gal4 / UAS-cAMP*; *Pdf-Gal4 / +* adult flies (green). Bottom: Response of LN_vs expressing cAMP to either 40 μM forskolin (red) or DMSO (blue). The data are an average of 12 LN_vs from three brains. (B) Top: Diagram showing an adult *Drosophila* brain and the approximate location of adult LN_vs expressing Epac-camps in *Pdf-Gal4 / UAS-Epac-camps 50A*; *Pdf-Gal4 / +* adult flies (light blue). Bottom: Response of LN_vs expressing Epac-camps to either 40 μM forskolin (red; n = 5 neurons from two brains) or DMSO (blue; n = 8 neurons from 3 brains). (C) Diagram illustrating

communication between larval LN_v and DN clock neurons. cAMP_r (Green) was expressed in all nine clock neurons per hemisphere using *tim(UAS)-Gal4* (5 LN_vs and 4 DN_s). The four PDF-expressing LN_vs were also programmed to express the mammalian purinergic receptor P2X₂ (orange circle). ATP stimulates the four LN_vs to fire and release the neuropeptide PDF (black dots), which bind to and activate the PDF receptor (PDFR, black circles) present on all larval clock neurons. Flies of the following genotypes were tested using two-photon imaging: *tim(UAS)-Gal4 / UAS-cAMP_r; Pdf-LexA, LexAOP-P2X₂ / + (Pdf > P2X₂); tim(UAS)-Gal4 / UAS-cAMP_r; + / TM6B*] (Control). (D) Left: Images of cAMP_r responses in larval DN_s before (Pre ATP) and after (Post ATP) the application of ATP. Images are in grayscale (top) and pseudocolor (bottom). DN_s were from larvae in which P2X₂ was expressed only in LN_vs and cAMP_r was expressed in all clock neurons. Right: Quantification of the cAMP_r responses in larval DN_s. Red: DN_s with P2X₂ in LN_vs and cAMP_r in all clock neurons. Data are from four neurons from three brains. Blue: DN_s from control larvae with no *Pdf-LexA* or *LexA-P2X₂* transgenes. Data are from 12 neurons from three brains. ATP (2.5 mM) was added during the time represented by the gray box. Data are plotted as described in Fig. 2B. (E) Left: Images of cAMP_r responses in larval LN_vs before (Pre ATP) and after (Post ATP) the application of ATP. Images are in grayscale (top) and pseudocolor (bottom). LN_vs were from larvae in which P2X₂ was expressed in LN_vs and cAMP_r was expressed in all clock neurons. Right: Quantification of cAMP_r responses in DN_s. Red: LN_vs from larvae with P2X₂ in LN_vs and cAMP_r in all clock neurons. Data are from 22 neurons from three brains. Blue: LN_vs from control larvae with no *Pdf-LexA* or *LexA-P2X₂* transgenes. Data are from 23 neurons from three brains. ATP (2.5 mM) was added during the time represented by the gray box. Data are plotted as described in Fig. 2B.

Fig. 6. Simultaneous measurement of Ca^{2+} and cAMP in whole brains

(A) Images of PDF-expressing LN_v cell bodies or projections before (0) or during (150 and 300 s) the treatment of larval brains with carbachol. Top: cAMPPr fluorescence in green and pseudocolor to measure intracellular cAMP. Middle: RCaMP1h in red and pseudocolor to measure intracellular Ca^{2+} . Bottom: merged cAMPPr (green) and RCaMP1h (red) fluorescence images to simultaneously show cAMP and Ca^{2+} . (B) RCaMP1h fluorescence from 12 PDF-expressing LN_v cell bodies and projections from four brains. Shading indicates SEM. (C) cAMPPr fluorescence from 12 PDF-expressing LN_v cell bodies and projections from four brains. Shading indicates SEM. (D) Data re-plotted from (B) and (C) to compare

the dynamics of changes in cAMP_r and RCaMP1h fluorescence in PDF-expressing LN_v cell bodies. (E) Data re-plotted from (B) and (C) to compare the dynamics of changes in cAMP_r and RCaMP1h fluorescence in PDF-expressing LN_v projections.